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APPLICATION NUMBER: *60/541,781*
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ER021168514US

31355 U.S. PTO 60/541781 020404

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Rolf	Halden	Baltimore, MD

Additional inventors are being named on the — *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Method for Environmental Monitoring and Bioprospecting

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number:

OR

☑ Firm or Individual Name	Johns Hopkins University				
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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 117
☑ Drawing(s) *Number of Sheets* ~~22~~ 22
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☐ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fees.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Heather Bakalyar

Date 2/4/04

REGISTRATION NO. 45,282 *(if appropriate)*

Docket Number: 4207

TELEPHONE 410-516-8300

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This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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U.S. Provisional Patent Application

JHU Ref. No. 4207

Method for Environmental Monitoring and Bioprospecting

Inventors:
Rolf Halden

Johns Hopkins University
Licensing & Technology Development LTD

Adendum to 4207

Report of Invention Disclosure Form (ROI)

This form is to be completed and submitted to the JHU office of Licensing and Technology Development (LTD) by anyone who believes they have developed a new invention. The purpose of this form is to enable LTD to evaluate whether legal protection to the invention will be sought and/or commercialization pursued. Please submit this form with all inventor(s) and Department Director(s) signatures. Visit the LTD web site at http://jhu.edu/technology/roi.html for HTML and Word downloadable formats of this form.

INVENTION INFORMATION

Title of Invention: Method for Environmental Monitoring and Bioprospecting (D-4207)

Name of Lead Inventor:	Halden	Rolf	Ulrich	Ph.D., P.E.
	Last	First	Middle	Degree

Lead Inventor Information: [The Lead Inventor is the primary contact person for LTD on all matters associated with this Report of Invention, including processing, patent prosecution and licensing. For reasons of administrative efficiency, it is the responsibility of the Lead Inventor to keep all other JHU inventors named on this Report of Invention informed of the status of such matters.]

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Are you a Howard Hughes Medical Institute employee or investigator? ☐ Yes ☒ X No

Are you a Kennedy Krieger Institute employee or investigator? ☐ Yes ☒ XNo

Additional inventors: ☐ Yes ☒ XNo If yes, please complete Additional Inventors section for each inventor.

LTD Internal Use Only: REF- 4207 TLA GHS Field of Use _____

All publications, patents and patent applications disclosed herein are incorporated into this application by reference in their entirety.

For example: "Sambrook et al, Molecular Cloning, A Laboratory Manual (volumes I-III) 1989, Cold Spring Harbor Laboratory Press, USA" and "Harlowe and Lane, Antibodies a Laboratory Manual 1988 and 1998, Cold Spring Harbor Laboratory Press, USA" provide sections describing methodology for antibody generation and purification, diagnostic platforms, cloning procedures, etc. that may be used in the practice of the instant invention.

INVENTION DESCRIPTION

Describe the invention completely, using the outline given below. Please provide an **electronic copy** of the invention disclosure document, references, and abstracts in Windows format on CD-ROM or floppy disk if possible

1. **Marketing Summary** [Please provide a non-confidential summary of the invention that can be used for marketing purposes. Unique details that are published may also be included.]

Brief Description:

For the management of contaminated sites, the risk assessment of microorganisms introduced into natural environments, and the search for novel microorganisms/enzymes/compounds applicable to biotechnology, a monitoring tool and analysis strategy are disclosed allowing for the automated, rapid and simultaneous determination of the following parameters: (1) water quality and toxicity, (2) intrinsic bioremediation potential, (3) accelerated bioremediation potential following nutrient amendment, (4) effective bioaugmentation strategies for environmental cleanup, (5) turnover rates of natural compounds and environmental pollutants under natural and enhanced conditions, (6) in situ DNA synthesis and protein expression, (7) in situ growth/death rates and metabolic activity of native and introduced biological agents under natural and altered environmental conditions, (8) structure and dynamics of microbial communities indigenous to natural soil and water environments, (9) identity and activity of microorganisms of potential value for use in biotechnology.

Potential Commercial Use:

The environmental monitoring tool and strategy could be sold as a license, product and/or service. The technology can be used to obtain in a one-step process a comprehensive assessment of contaminated waste sites based on which treatment strategies can be selected, implemented and then monitored, again using the new technology. The invention may be applied to assess the potential risk resulting from the release of pathogens and genetically engineered microorganisms into natural environments. In addition, it has potential value for discovering microorganisms, enzymes and natural products of relevance for the pharmaceutical industry and the biotechnology sector.

Marketing Goal:

Johns Hopkins University is seeking licensees for this technology

Keywords:

Bioremediation, environmental management, site assessment, risk assessment, bioaugmentation, bioprospecting, water quality, toxicity, contaminants, bioterrorism, pathogens, environmental monitoring.

SOFTWARE –Does this disclosure include a software element or software is implemented in the invention ☐ Yes ☐ No

If yes, please complete the Software Information Form which can be found at: _____

BIOLOGICAL MATERIAL – Does this disclosure include biological material, ☐ Yes ☐ No

If yes, please attach a list of materials for reference. A Tangible Property Report of Invention form may be completed if the disclosure is biological materials only. You can find this form at: http://www.hopkinsmedicine.org/lbd/otl/

2. Problem Solved [Describe the problem solved by this invention]

The new tool and analysis strategy allows one to determine the microbial community structure of complex environmental mixed cultures, to link an observed chemical, biochemical and/or physical change to a particular microorganism, to study microbial interactions, and to culture and study previously uncultivated microorganisms in pure culture and during interaction with their natural environment. The device and technology can be applied in situ and ex situ (on-site or in the laboratory). Due to the incubation of the tool in situ, rates and metabolic activities determined with the device are expected to closely mirror actual actions currently occurring or potentially occurring *in situ*. In addition, the device may be inoculated in situ and incubated and analyzed ex situ. Alternatively, inoculation and incubation of the device can be performed ex situ. The use of isotopes in conjunction with molecular-genetic and/or proteomic analysis techniques allows one to distinguish dead and dormant microorganisms from metabolically active ones (only viable cells will incorporate isotope labels into biomarkers). Parallel testing of effects caused by various environmental parameters (*e.g.*, type and concentration of added nutrients/mixtures/microorganisms) allows one to deduce which of the metabolically active microorganisms are responsible for an observed change. This has important implications for the design and monitoring of bioremediation strategies, e.g. bioimmobilizaiton of uranium by bacteria, or the dechlorination of toxic chloroethenes, etc. Taken together, these characteristics of the new technology provide a hitherto unattained level of discriminatory power that will enable one to selectively enrich for, culture and identify novel microorganisms and microbial functions. This is of great importance for the cleanup (bioremediation) of contaminated sites and for the biological prospecting for novel microorganisms, biomolecules, drugs and metabolic processes. Furthermore, the technology can be used for the in situ cultivation of microorganisms that do not grow in the laboratory, and for assessing the survival and metabolic activity of foreign species in natural environments, which is of importance to public health.

3. Novelty [Identify those elements of the invention that are new when compared to the current state of the art]
The tool and analysis strategy are novel because they allow for the first time the cultivation and comprehensive biochemical characterization of microorganisms in their natural environments. The technology is novel in that is combines in a non-obvious fashion the following tools/approaches: solid-phase sampling techniques, in situ enrichment and biochemical screening, use of electron donor/acceptor pairs, isotope labeling and massive parallel screening with automated analysis. The technology is novel in that it provides data for hundreds or even thousands of hypothetical environmental scenarios, thereby allowing one to determine quickly and in an automated fashion the likely rates of environmental change induced by these perturbations. The strategy is novel in that it makes use of in situ microcosm arrays in conjunction with culture-independent microbial community analysis to obtain a comprehensive picture of microbial communities. It is suitable for linking specific microbes to observed reactions by using computer-assisted subtractive profiling techniques. It is fully compatible with existing robotic systems thereby allowing for rapid and fully automated analysis using chemical, physical, biological, genomic and—more importantly—proteomic analysis techniques. The proposed inclusion into the in situ microcosm array sampler of miniaturized pumps, closure mechanisms, semi-permeable membranes and filters is new as it will allow one to first inoculate and then incubate the device in the environment without removing (and potentially harming) the resident microbes from their natural environment. The device can be equipped with microfluidic systems allowing for delivery of small volumes and defined quantities of microorganisms to the test chamber prior to physical and/or chemical containment of the captured specimens via barriers that are either non-permeable, semi-permeable or completely permeable for chemical compounds; this aspect will allow one to culture uncultivated or "non-culturable" bacteria to numbers sufficiently large to perform biochemical characterization and identification. The technology is suitable for determining the rate of protozoan grazing in situ. The device also allows one to determine how non-native microorganisms will cope in natural environments when confronted with physical, biological and/or chemical stressors. For this application, test organisms will be inoculated into the device prior to its deployment. Semi-permeable membranes can allow the introduced species to come into contact with the target environment while staying contained in the device. Inoculation of some of the test chambers with known quantities of test microorganisms also can assist in determining the toxicity of a natural environment and in normalizing assessment data for direct comparison of geographically distinct environments.

4. Potential Commercial Use – [What products can be produced with this invention.]
Potential Commercial Use:

The environmental monitoring tool and strategy could be sold as a license, product and/or service. The technology can be used to obtain in a one-step process a comprehensive assessment of contaminated waste sites based on which treatment strategies can be selected, implemented and then monitored, again using the new technology. The invention may be applied to assess the potential risk resulting from the release of pathogens and genetically engineered microorganisms into natural environments. In addition, the invention can be use to discover microorganisms, enzymes and natural products of relevance for the pharmaceutical industry and the biotechnology sector.

JHU REF: _____

8. Workable Extent/Scope [Describe the future course of related work, and possible variations of the present invention in terms of the broadest scope expected to be operable; if a ***compound,*** describe substitutions, breadth of substituents, derivatives, salts etc., if ***DNA or other biological material,*** describe modifications that are expected to be operable, if a ***machine or device***, describe operational parameters of the device or a component thereof, including alternative structures for performing the various functions of the machine or device]

The proposed technology has a broad workable extent. The instrumentation and analysis technique can be optimized for exclusive in situ applications, ex situ applications, or a combination of the two. Modification of the closure mechanism configuration described on the attached pages will allow for sequential opening and closing of microcosm compartments. Real-time and monitoring equipment can be added to the device to increase functionality and to trigger reactions at specific points in time selected by changes in the target environment (e.g., heavy rainfall events). Use of radio frequency signaling and remote controls can replace the umbilical cord shown in the attached Figure. The design of the device can be altered to allow dployment of the device in environments featuring extreme conditions including, but not limited to, extreme pH, temperature, pressure, radiation, etc. Microfluidics, filters of varying sizes, semi-permeable membranes and alternative closure mechanisms may be integrated into the sampler to separate in time the inoculation of the device from the incubation period that allows chemical change to take place within the sampler. Optical and/or electrical detection systems may be incorporated in microfluidic configurations to seal individual microcosms as soon as a single cell has been delivered to the microcosms, thereby greatly increasing the success rate of isolating novel microorganisms. Proteomic approaches may be used for rapid and fully automated analysis (e.g., matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and protein sequencing of enzymatic digests using tandem mass spectrometry (MS/MS). Central facilities may be used for analyzing samplers deployed in situ. This will allow for automated analysis and for a high degree of standardization. Standardized analysis in turn will dramatically improve measurement precision and will allow one to determine the systematic biases of the technique (due to "bottle effects") that may limit measurement accuracy; once identified, these biases can be accounted and corrected for thus enabling one to predict—with high accuracy and precision—the environmental change to be observed following engineering interventions. For bioremediation purposes, this would entail the development of databases that record predicted biotransformation rates and rates actually observed in situ. The format of the tool allows for automated analysis. Speed and ease of analysis may be achieved by replacing molecular-genetic analyses with other more convenient measurement techniques suitable for discerning isotope distributions (e.g., use of MALDI-TOF MS and bioinformatics database searches for automated microorganism identification). Sample processing using commercially available robotics (e.g., Amersham Biosciences robotics) and tools for rapid sample cleanup and processing (e.g., Gyrolab MALDI SP1 etc.) in conjunction with enzymatic digestion steps (e.g., trypsin digestion).

The device also may be adapted for studying the fate of either beneficial or hazardous biological agents in natural environments. This work would require the device to be modified to reflect as closely as possible within each test compartment the physical/chemical/biological environment of interest (e.g., flow-through cells equipped with local sediment etc.). Again, the device would be equipped with a semi-permeable barrier allowing for interaction of the test species with the environment without allowing for its release.

9. References [Please cite relevant journal citations, patents, general knowledge or other public information related to the invention and distinguish between references that (A) contain a description of the current invention from those that (B) contains background information.]

A

Halden, R.U. Innovative Method for Environmental Monitoring and Bioprospecting. The Forth International Conference on Remediation of Chlorinated and Recalcitrant Compounds. Monterey, CA, May 24-27, 2004.

B

Jeon CO, Park W, Padmanabhan P, DeRito C, Snape JR, Madsen EL. Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. Proc Natl Acad Sci U S A. 2003 Nov 11;100(23):13591-6. Epub 2003 Nov 03.

Geyer, R., A. D. Peacock, Y.-J. Chang, Y.-D. Gan, and D. C. White. 2002. Presented at the 2002 NABIR PI Conference, Arlie, VA. Down-Well Microcosm "Bug Traps" and Subsurface Sediments for Rapid expanded-Lipid-Biomarker Analysis and DNA Recovery for Monitoring Bioremediation Microbial-Community Ecology within Samples from Uranium-Contaminated Sites. In 2002 NABIR PI Conference. 2002. Arlie, VA.

Nayar, S., B. P. L. Goh, L. M. Chou, and S. Reddy. 2003. In situ microcosms to study the impact of heavy metals resuspended by dredging on periphyton in a tropical estuary. Aquatic Toxicology 64:293-306.

Zengler, K., G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller. 2003. Cultivating the uncultured. PNAS.

Short, J. M., and M. Keller. 2001. U.S. Patent 6,174,673

Connon, S. A., and S. J. Giovannoni. 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates.

☐ No references available at this time.

Description of the Technology

Configurations of the microcosm array for bioremediation and bioprospecting in addition to those mentioned in the attached research proposals; in situ and ex situ applications.

In addition to the details provided in the attached research proposals, the device can be equipped/used as stated below:

Ex situ application. The ability of the device to carry out a large number of experiments under controlled conditions in a standardized and efficient format can also be exploited using ex situ techniques. For example, the device may be deployed in situ for inoculation and incubated ex situ (in the field or in the laboratory) prior to analysis. Alternatively, a liquid sample may be obtained from a given target environment and the device may be inoculated ex situ and incubated ex situ.

Mechanism for sequential closing of individual microcosms. The closure mechanism shown in the attached pages can be reconfigured to allow for sequential closing of individual microcosms. Separation of the valve plate into sections covering individual rows will allow to selectively close one row at a time. Placement of identical microcosms in different rows of the device will allow to collect multiple samples of the replicate experiments at various discrete time points. In this way, biochemical activity and chemistry can be measure at different points in time to facilitate time-discrete measurements of and to allow for various degrees of chemical labeling when employing isotopic labels.

Addition of (real-time) monitoring probes to the device. The utility of the device may be further enhanced by adding instrumentation for environmental monitoring to the device. Monitoring probes and sensors may be integrated into the device or added on to its exterior or immediate vicinity. Parameters suitable for monitoring and real-time monitoring include, but are not limited to: pH, conductivity, redox potential (Eh; ORP), temperature, salinity, alkalinity, turbidity, dissolved oxygen, dissolved oxygen saturation, chlorophyll, pressure, depth, particle concentration, particle size, etc. Use of particle counters in conjunction with the microcosm array will be particularly beneficial when attempting to deliver a predefined quantity of microorganisms into specific chambers. Signals of the above probes and sensors may be transmitted through cables, circuits, radio waves or other media suitable for signal transmission. For off-line monitoring and long-term monitoring, collected information may be stored in place (within the device) or remotely (e.g., in the field or in the lab).

Delivery of chemicals to stop chemical reactions and microbial activity. In order to measure the extent of chemical reactions taking place in the device, reagents may be delivered to individual microcosms at different points in time to abort reactions and prevent further chemical change. This feature will be beneficial for the determination of reaction kinetics as it will allow to obtain "snapshots" of the chemistry in individual microcosms over time. Chemical modifiers suitable for stopping on-going reactions include, but are not limited to, pH agents (acids, bases), selective enzyme inhibitors (e.g., acetylene gas for inhibition of oxygenases), heavy metals binding to enzyme complexes (e.g., mercury chloride) and others. Chemical modifiers may be delivered passively or actively. Delivery modes include, but are not limited to, injection from a pump,

pressurized vessel or equivalent, diffusion into the microcosm from a sorbent material, rapid heating or cooling of the device or chamber, and equivalent techniques.

Sorbent materials for chemical analysis. Effluent from the individual flow-through microcosms will be passed through a sorbent material (e.g., chromatographic columns, C-18 solid-phase-extraction plates [Spec manufactured by Varian], ion exchange cartridges, disk filters, membranes or other) to sorb and capture selected chemicals/specimens of interest. Following retrieval of the device, chemicals/specimens collected on the sorbent can be removed and analyzed. This allows one to conduct a complete mass balance on microorganisms and chemicals entering and leaving the device. For this purpose, sorbent arrays can be located downstream and/or upstream of the microcosm array.

Individual collection vessels. The attached drawings shows a single receptacle for the combined effluent of all flow-through microcosms. Alternatively, the effluent of the individual microcosms may be collected separately. Thus, the single effluent bottle shown in the attached research proposal may be replaced by a manifold connected to hundreds or thousands of small bladders that can capture the effluent from each individual microcosm thereby providing an absolute mass balance on all materials that passed through each of the microcosms.

Standardized microcosms. Each ISMA can contain a number of "standardized microcosms." The latter are flow-through microcosms containing a known quantity of well-defined microorganisms and varying amounts of test compound(s) (none to high concentrations). Standardized microcosms will provide a measure of the toxicity of the test environment. In addition, analysis of the survival and growth of these microorganisms and their metabolic activities under the respective conditions will allow one to normalize test results for ISMA samplers deployed in different locations and at different points in time.

Integration of filters in selected microcosms. Selected microcosms will be equipped with a filter (placed at the inlet or further upstream of the flow-through microcosm). Filters will allow one to selectively exclude certain microorganisms from entering a flow-through cell. For example, exclusion of protozoans will allow one to determine the rate of protozoan grazing by comparing the results of two sets of microcosms that were identical except for the presence of the filter in one set of the systems. Similarly, one may exclude larger bacteria using a particular pore-size filter to selectively enrich for small bacteria (micro- and nanobacteria).

Modifiers. Selected flow-through microcosms will be equipped with chemical inhibitors, inducers and similar chemical modifiers. This will allow one to selectively induce the expression of proteins and metabolic functions of interest in captured microorganisms. In addition, chemical modifiers may be used to selectively suppress subpopulations within the device. For example, antibiotics can be included to suppress growth of fungi, certain bacteria and protozoa. Similarly, inhibitors can be included to prevent the growth and activity of microbial subpopulations; for example, sulfate reducing bacteria and methanogens may be selectively inhibited using sodium molybdate and BES, respectively. Additional selective inhibitors exist that inhibit other subpopulations.

Test compound delivery system. Agar is only one of many substances to be used as a substratum for microbial colonization and as a medium for continuous release of test compounds. Alternative materials include gellan gum with or without CaCl (Jansen et al. 2002), and other inert materials such as glass or plastic that can be molded to form columns, porous networks, beads, etc. Test compounds may also be presented to microorganisms within the microcosms using solids (crystals) and coatings of poorly water-soluble compounds (nonaqueous phase liquids). Gases may be delivered to the system using any of the following techniques: in situ generation of the desired dissolved gaseous species; adsorbed gases; semi-permeable membrane vesicles filled with gasses of interest (passive gas delivery); or active gas delivery using miniaturized pressurized gas bottles.

Pumps. The test medium (e.g., groundwater or seawater) may be delivered by a single pump using conventional pump systems such as centrifugal pumps, rotary pumps, piston pumps, syringe pumps (twin configuration; one syringe delivers while the other is being filled), peristaltic pumps and/or bladder pumps. Alternatively, multiple pumps may be used to achieve similar flow rates in all systems regardless of pressure buildup that may occur as a result of in-line filters, physical clogging and microbial growth. These pump arrays may use any of the above pump mechanisms or equivalent others.

Microfluidics and multiple arrays. Some applications may benefit from the use of microfluidics and operation of multiple arrays in parallel or in series. For example, for bioprospecting studies the number of test compartments may be as high as several thousand per microcosm array. These miniaturized systems will be fed with test medium (groundwater, seawater, etc.) using microfluidic systems that minimize dead volume within the device and allow for delivery of (sub-)microliter quantities of test medium to the individual microcosms. This configuration will be ideal for bioprospecting studies to cultivated otherwise "non-culturable" microorganisms. Following delivery of a small volume of test medium to a microcosm, the valve plates may move into an intermediate position thereby placing a semi-permeable membrane at the entrance and exit of a microcosm. Thus, individual microbial cells may be trapped randomly in a single microcosm. These may then be incubated in a flow-through mode that allows chemicals to enter and exit the test volume while microorganisms are prevented from moving in or out of the test vessel. In this way, confined microorganisms may be cultured while still being in "chemical communication" and interacting with other microbial community members. Cell signaling and other chemical interactions occurring only in situ are known to be essential for certain microorganisms to proliferate. Operating thousands of microcosms in one array and several of these arrays in parallel or in series will facilitate high throughput screening of large numbers of organisms under diverse test conditions.

Adaptation of the device for deep-sea exploration. Using the configuration and materials shown in the attached drawing, the device is estimated to withstand depth of 100 meter and below. Alternative materials such as the use of stainless steel with or without polymer coating will make the device suitable for deep-sea deployment. The umbilical shown in the attached figure may be replaced with a remote and/or programmable control mechanism.

Combined use of the ISMA with a flow cytometer. Capturing of individual microbial cells for in situ or ex situ cultivation may be attempted by chance, that is, by delivering to the microcosm of

a small volume of liquid likely to contain only one or a few microbial cells. This somewhat inefficient process will be greatly enhanced by using the microcosm array in conjunction with a flow cytometer. The advantage of this configuration is that individual cells and microcolonies can be directed to, and sealed in, individual microcosms. Deposition of known quantities of cells will be advantageous for increasing capture efficiency and cultivation efficiency. In addition, the flow cytometer could be used to distinguish particles thereby selectively "harvesting" cells of interest while diverting those that have a low likelihood of representing unique clones. Containment of microbial cells of interest can be complete—via use of the solid valve plate or equivalent—or selective—via use of a semi-permeable membrane allowing for both physical isolation of cells and continuing "chemical communication" with the surrounding environment.

JOHNS HOPKINS
BLOOMBERG
SCHOOL of PUBLIC HEALTH

In Situ Microcosm Array (ISMA) Technology

...an innovative technology for bioremediation, bioprospecting and environmental risk assessment

Rolf Halden, PhD, PE

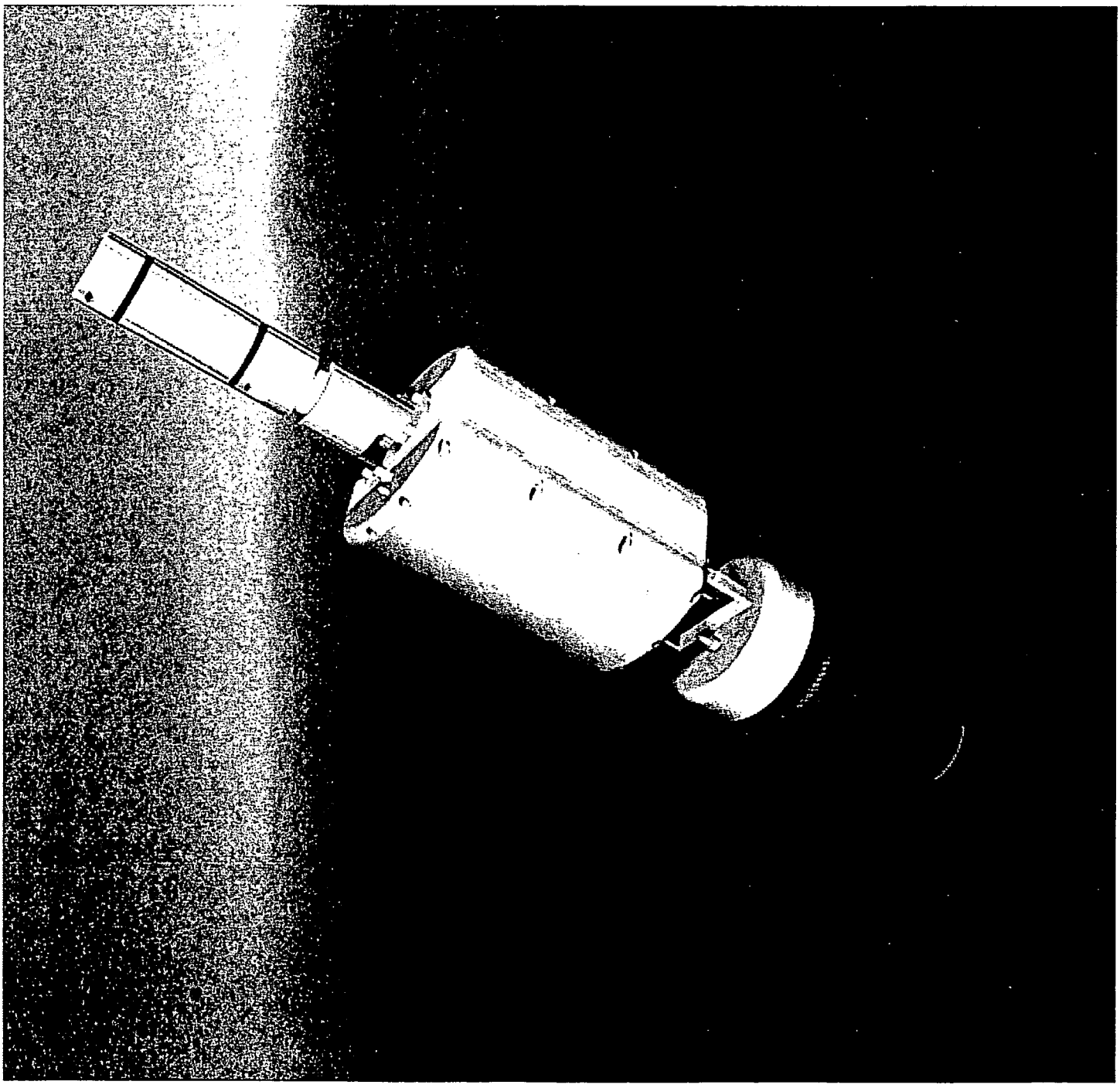
October 28, 2003

Outline

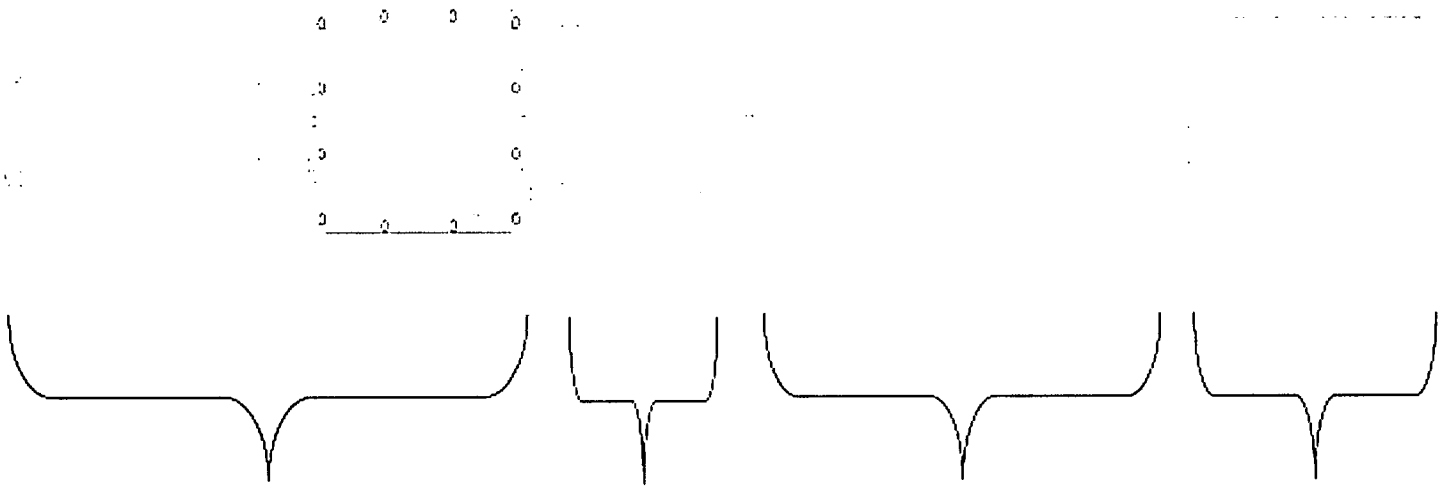
Technology Overview

- How it works
- Areas of Application
  - Bioremediation
  - Bioprospecting: Microbes/Enzymes/Natural Products
  - Risk Assessment
  - Robotic Analysis and Data Output

Market Analysis



ISMA System Components



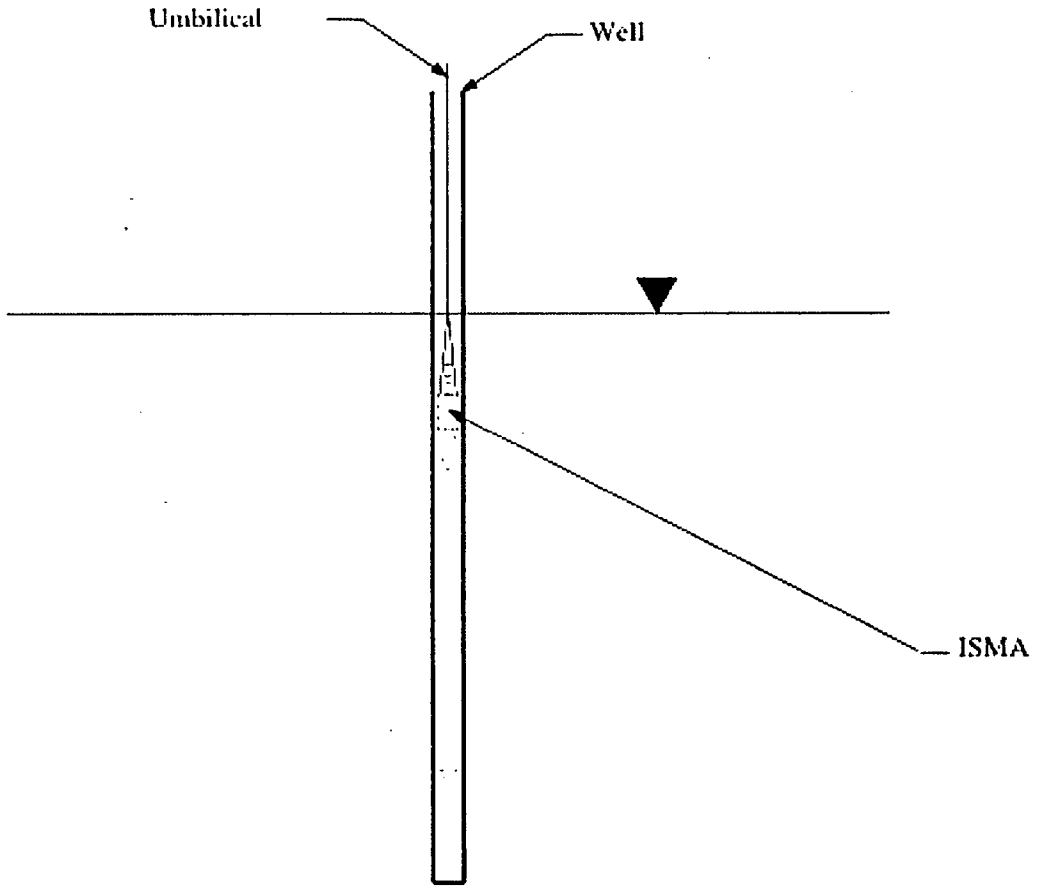
Array and Closure Mechanism

Pump

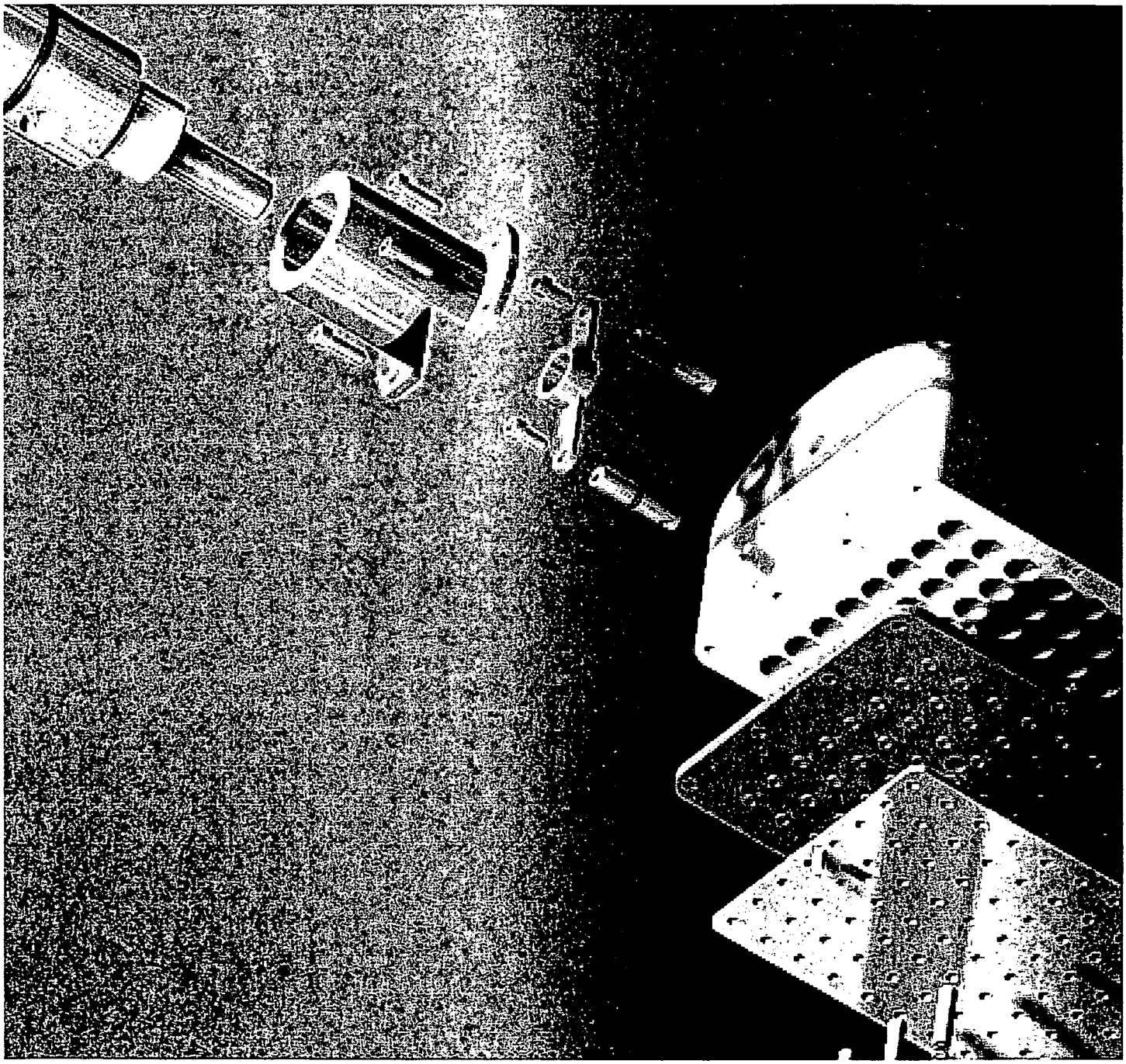
Effluent Bottle

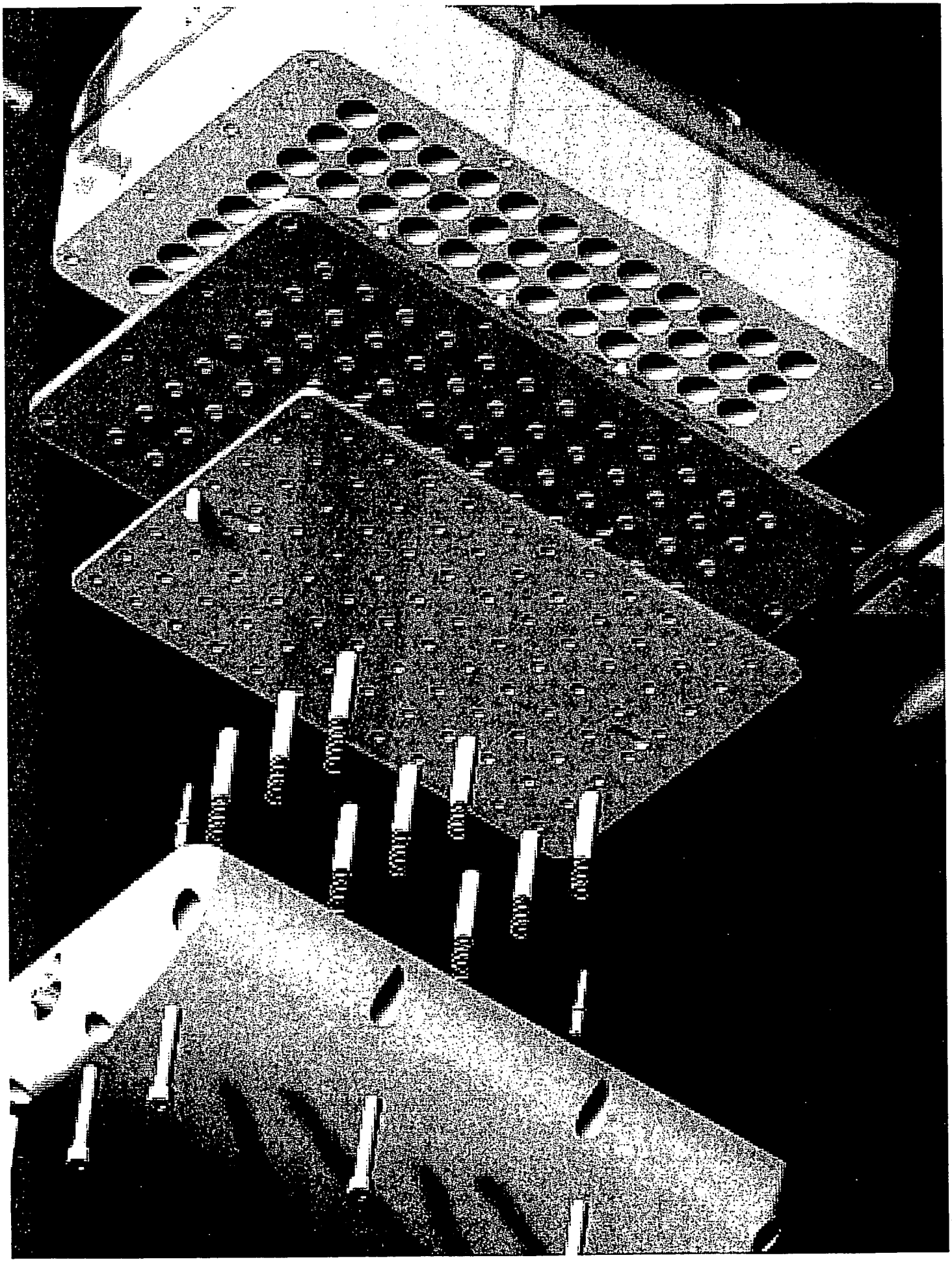
Weight

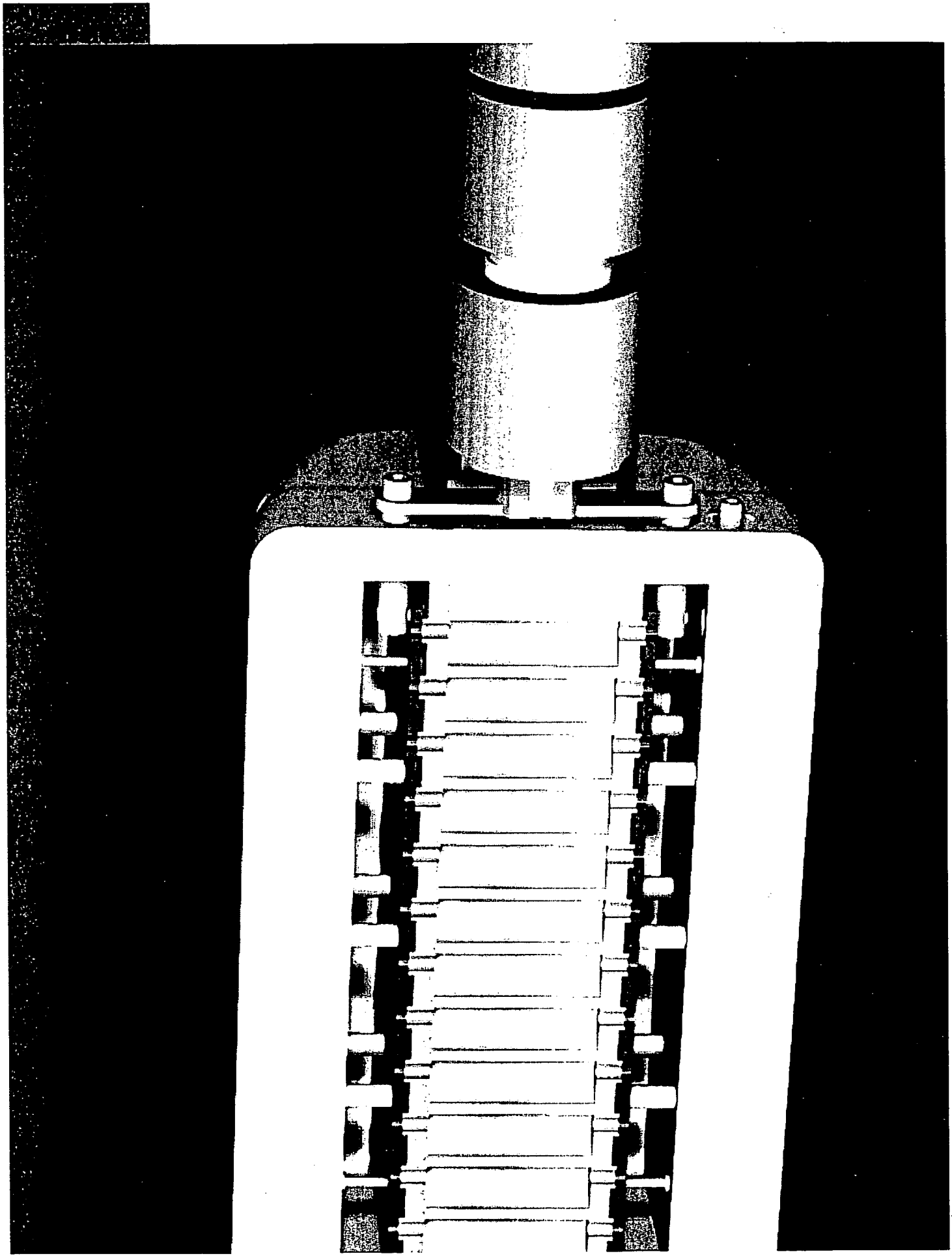
ISMA Deployment

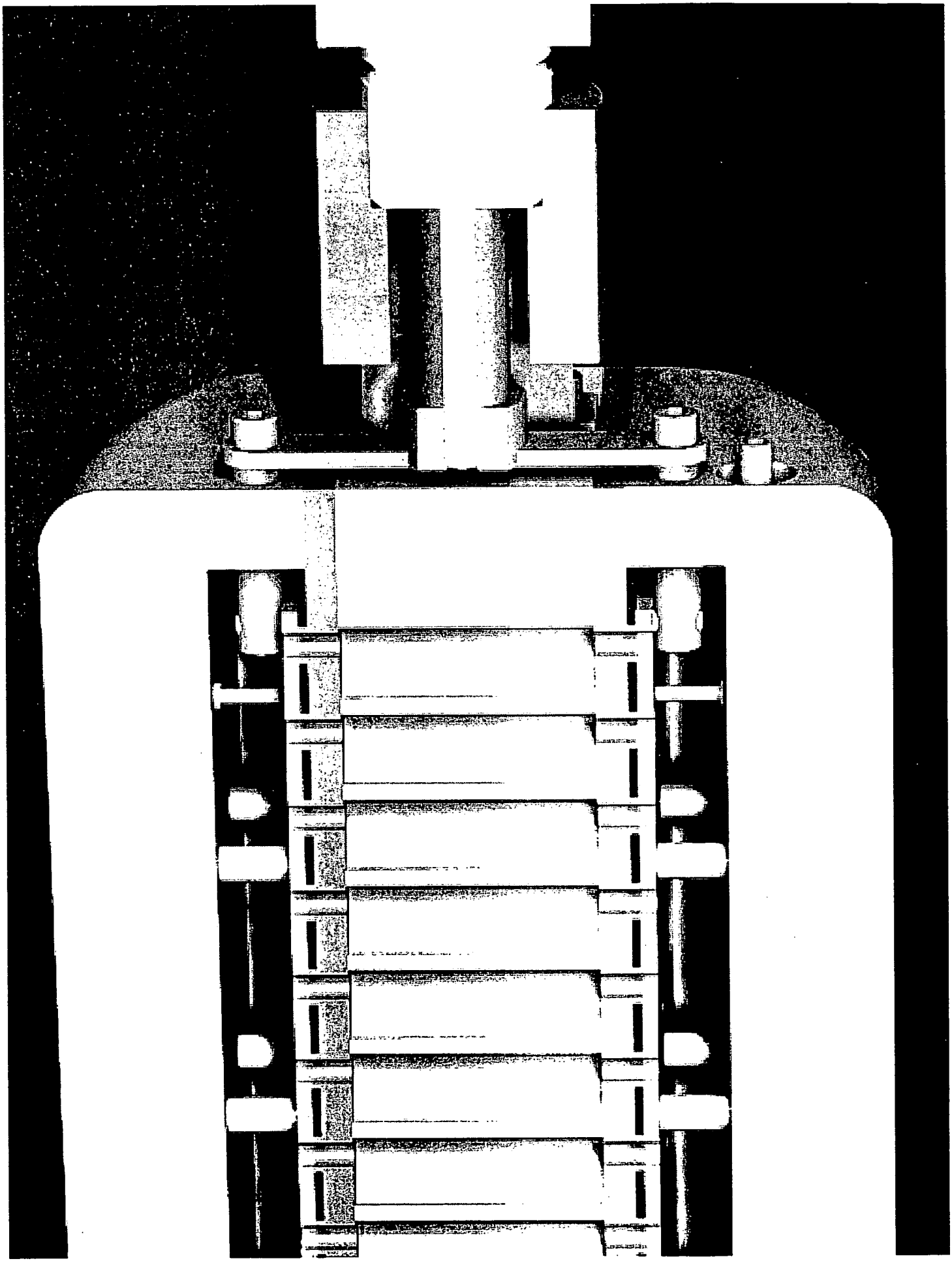


Schematic showing the ISMA suspended in a standard 100 mm diameter well. The device is supported from the surface via an umbilical, which supports the device and provides power and telemetry to the surface.



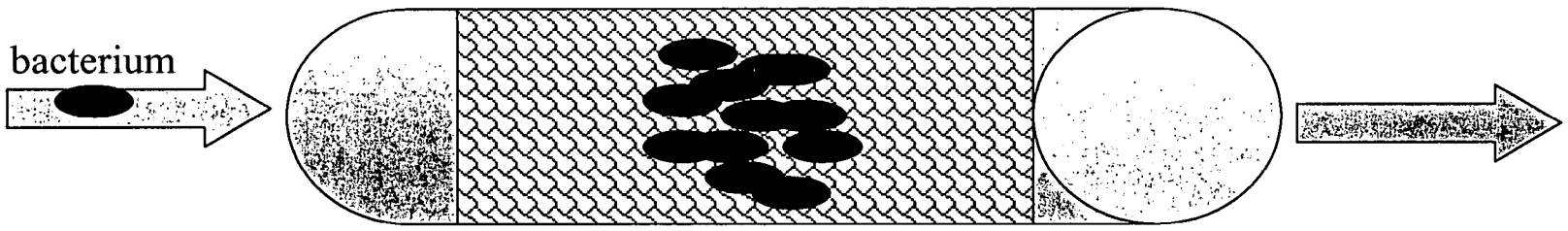






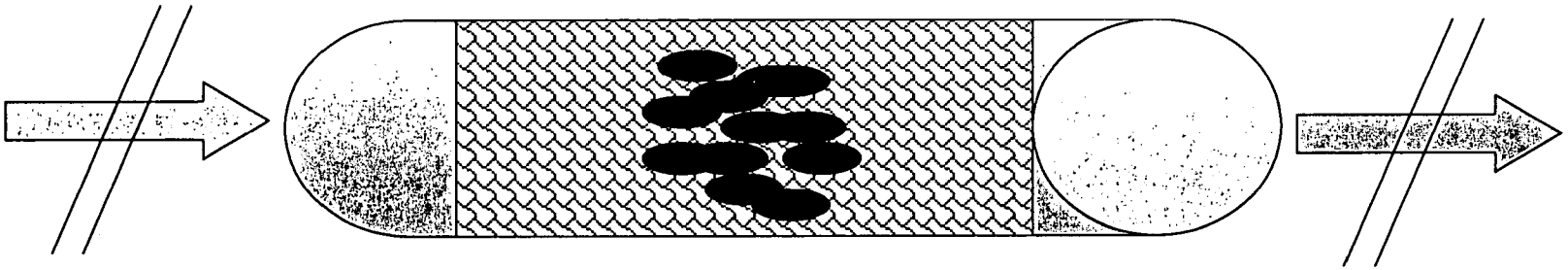
• Capturing Microorganisms

Concentrating microorganisms indigenous to natural waters



2. In Situ Incubation

Incubation in batch mode…

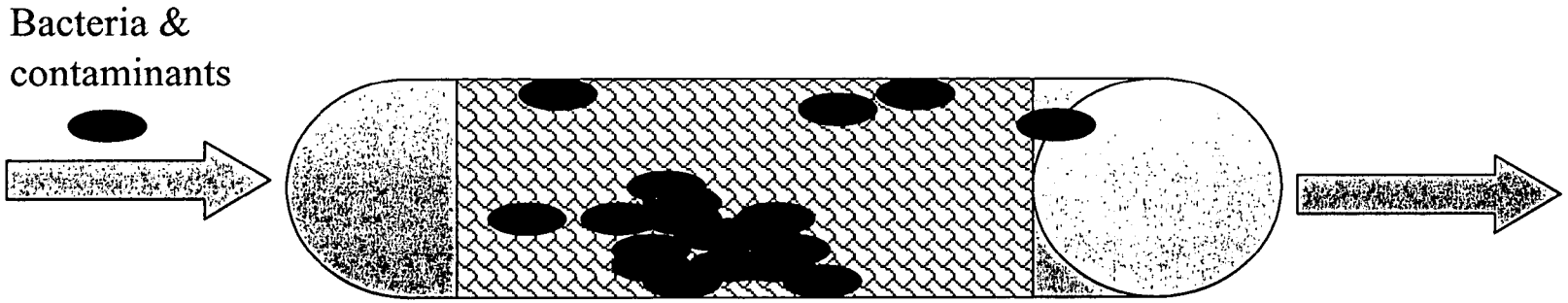


or containment of captured microorganisms
using a semi-permeable membrane

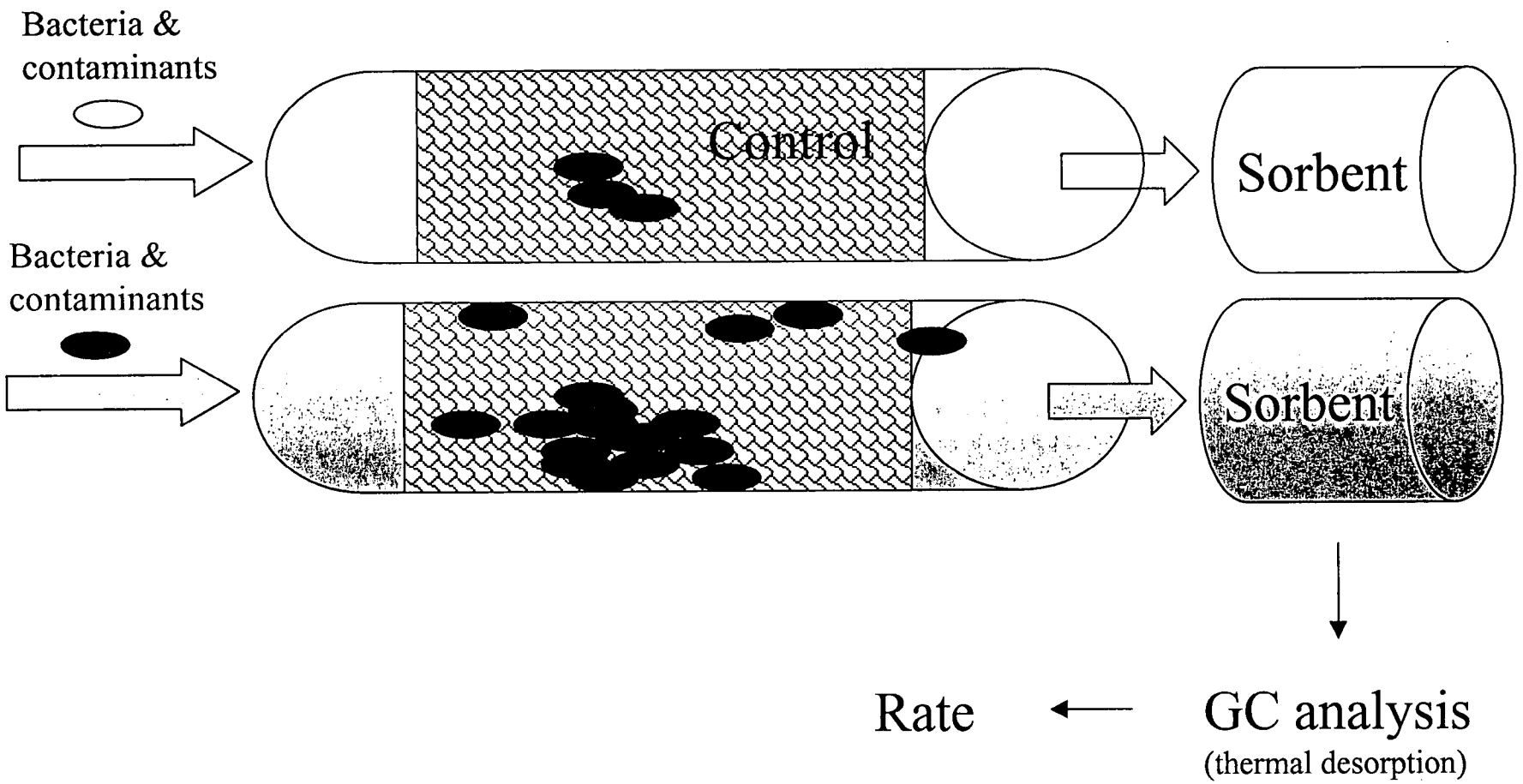
2. Selective Enrichment

Use controls containing no substrate to distinguish growth from filtration

3. Bioaugmentation

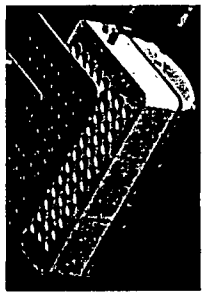


4. Biotransformation Rates



Robotic Analysis & Data Output

Automated Analysis



Genomic analysis

Proteomic analysis

Chemical analysis

Computational data analysis

Output

- New species
- New activities
- Biochem. rates
- Survival
- Standardized data
- Others

Bioremediation

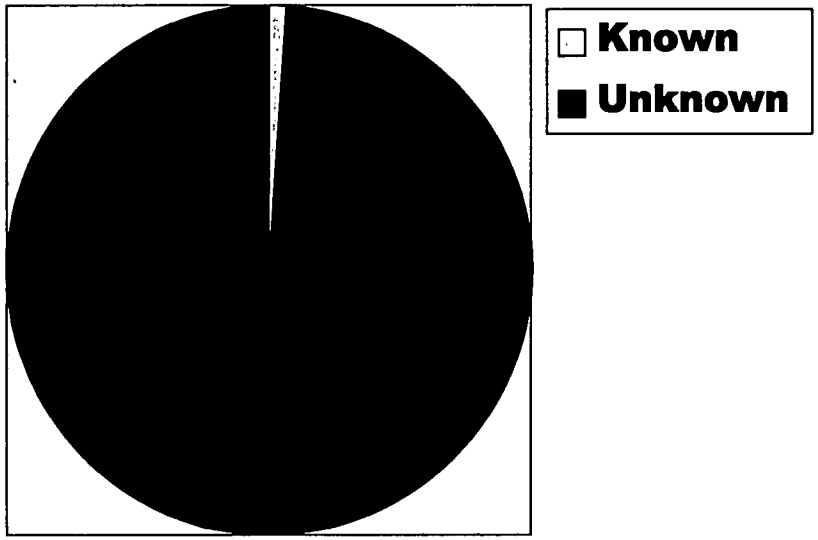
- Use of microorganisms to transform toxic environmental contaminants to non-toxic (or less mobile) compounds
- Each site is unique => customized bioremediation strategies
- Typical approach:
  - Microcosm experiments to determine
    - Intrinsic biodegradation rates
    - Enhanced degradation rates (nutrient addition)
    - Survival and activity of introduced bacteria (bioaugmentation)
  - Microbial community analysis to
    - Determine whether the right microbes are present
    - Convince regulators that contaminants are being degraded
  - Pilot-scale tests
  - Full-scale remediation

ISMA

Bioprospecting

Discovery of new microorganisms, enzymes, antibiotics etc. of potential value for the biotech and pharmaceutical industry

Microbiology 101



>99% of environmental microorganisms cannot be cultured in the laboratory. We do not know:

- Who they are
- How they make a living
- Why they refuse to grow in the test tube

Majority of biodiversity still untapped!

Environmental Risk Assessment

- Use of ISMA to determine any of the following parameters:
  - Water toxicity
  - Microbial survival/mortality in situ
  - Microbial metabolism in situ
  - Microbial growth in situ
  - Influence of chemicals on microbial ecology
  - Influence of introduced species on microbial ecology
  - Fate/activity of genetically engineered microorganisms in situ

In Situ Microcosm... Why?

- Transfer of groundwater/seawater to the surface affects:
  - Physical/chemical characteristics of water sample
    - Temperature change
    - Pressure change
    - Solubility of gases: e.g., loss of CO_2
    - pH change
    - Loss of chemicals due to oxidation, precipitation, sorption etc.
  - Activity, metabolism and fitness of microorganisms
- Therefore, in situ tests yield more reliable information

ISMA facilitates...

96, 384, 1536 (or more) experiments simultaneously

- Fully automated analysis
- Rapid analysis using MALDI-TOF MS & proteomics
- Comprehensive bioremediation design studies
- Use of toxic compounds in situ

  Measurement of in situ rates

  Integration of internal standards to directly compare hazmat sites

  Testing survival of GMOs and pathogens in situ

  Detection of specific metabolic activities via isotope labeling
- Use of centralized facilities increases data quality, allows for use of high-end equipment, eliminates experimental bias, allows for generating a database that links ISMA patterns to remediation histories of similar sites

Market Analysis

A commercialization report was completed by an independent consultant and indicates that the technology has potential for at least two markets:

- Bioprospecting
- Bioremdiation

Additional areas of application include:

- Environmental Risk Assessment

Down-Well Diagnostic Device for Environmental Monitoring and Bioprospecting

Rolf U. Halden, Ph.D., P.E., Assistant Professor of Environmental Health Sciences

Introduction

Bioremediation is an effective, yet inexpensive biotechnology for removing organic and inorganic pollutants from contaminated environments (Lowe, Madsen et al. 2002). When targeting dissolved metals and radionuclides, the goal is to convert water-soluble, toxic species to insoluble, less toxic daughter products (Loveley 2002). For example, uranium may be removed from contaminated groundwater and immobilized in the subsurface via the injection of carbon sources that stimulate the microbially induced precipitation of dissolved U(VI) in the form of insoluble U(IV). In this case, the contaminant is being treated "in place" and the process is being referred to as *in situ* bioremediation.

When designing *in situ* bioremediation strategies, it is essential to gain an understanding of the type, activity, and nutritional requirements of subsurface microbial communities present at a specific cleanup site (Halden, Tepp et al. 1999). Microbial community information also is important for convincing regulatory agencies and stakeholders that the contaminant is being removed (or, in the case of metals, successfully immobilized in the subsurface) rather than being diluted or dispersed in groundwater.

Currently, the assessment of bioremediation potential at a given site is a two-step process involving:

(1) DNA extraction and culture-independent profiling of microbial communities using organism-specific 16S rDNA sequences as "microbial name tags."

(2) Extensive microcosm screening studies to determine which substrates are suitable for eliciting and promoting a desired degradative, microbial function. In addition, these lab experiments yield estimates of contaminant removal rates which, unfortunately, often poorly reflect the actual kinetics occurring *in situ*.

This ___ __ describes a new technology that promises to accomplish both of the above tasks in a one-step process, yielding superior results by providing more detailed information of higher accuracy in a shorter time period at significantly reduced costs. The technology will yield information on what types of organisms are present, which of these are alive and metabolically active, which nutrients may be added to accelerate bioremediation, at which concentrations these additives should be used, which pollutants will be biodegraded and at what *in situ* rate the bioremediation process will proceed.

In addition, the technology may be used for biodiversity prospecting (bioprospecting), *i.e.*, it may be applied for the identification of useful microorganisms, metabolic processes, or products in nature (Lowe, Madsen et al. 2002).

Technology Description

The bioremediation of contaminated sites requires the development of automated, field-ready technologies for studying the complex microbial communities indigenous to contaminated subsurface environments. In order to address this need, a miniaturized down-well device has been invented that will facilitate (1) in-well microbial sampling, (2) culture-independent characterization of microbial communities of groundwater, (3) identification of metabolically active community members participating in the biotransformation of contaminants, (4) determination of bioremediation potential at the contaminated site, (5) identification of electron donor compounds suitable for stimulating pollutant-degrading communities, (6) identification of electron acceptor compounds used *in situ*, and (7) determination of the kinetics of *in situ* contaminant removal both under present conditions and under enhanced conditions. Although the technology potentially will be applicable to all organic and inorganic contaminants in most environmental media, the technical design presented in the following specifically targets the bioremediation of saturated subsurface environments containing metals and radionuclides. Uranium was chosen for illustrative purposes.

Biological reduction of U(VI) to U(IV) can be performed by a significant fraction of metal-reducing bacteria indigenous to subsurface environments (Loveley 2002). The presence of biotransformation potential does not

necessarily mean that bioremediation is or will occur, however. So how does one tell whether and which of the potentially relevant microorganisms detectable at a given site are performing the desired reaction? Currently available detection techniques generally do not provide a satisfactory answer to this question. The reason for this lies in the fact that in most field situations uranium-transforming microorganisms represent only a very small fraction of the microbial subsurface community. Since uranium represents only a small portion of the sum of electron-acceptors available *in situ*, uranium-transforming microorganisms are likely to be out-numbered by other indigenous bacteria. Thus, non-selective techniques such as amplification of 16S rDNA followed by denaturing gradient gel electrophoresis (DGGE) analysis may fail to detect uranium-reducing bacteria that are present at low densities only; the use of genus-specific PCR primers in contrast should always produce positive results when targeting ubiquitous microorganisms, irrespective of the organisms' actual role in the transformation of radionuclides. The use of stable isotopes as chemical reporters can help to discriminate metabolically active bacteria from dormant or dead community members and from those performing functions unrelated to bioremediation/biostimulation.

I propose the use of stable-isotope labeled substrates (*e.g.*, ^{13}C-labeled acetate) as chemical reporters of biotransformation activity in miniaturized, field-deployable down-well devices. The device will have identical dimensions to a 96-well microtiter plate (or to other commercial test systems for which automated analysis already has been perfected). The device will be deployed—folded into the smallest possible conformation—by lowering it into a standard casing of a groundwater monitoring well (typical well casing diameter is 4 inches). Each of these tools will hold 96 (and potentially even more) different, physically separated test environments, or "test wells."

Once the device has been lowered into the monitoring well to the desired depth, it will be triggered from the surface via an electrical signal conducted by a wire (or via other means such as a programmed build-in mechanism). Triggering of the device will expose each of the up to 96 "test wells" to the flow of groundwater. Microorganisms suspended in the groundwater will attach themselves to the presented surfaces and will become trapped in the device. Additional free-living microbes will become trapped once the device receives the signal to close again. Some of the test wells will have uranium-coated bottoms (representing the contaminant of concern). Individual test wells may also contain one stable-isotope labeled nutrient for determining its effect on microbial growth and activity. The now closed device will be incubated *in situ* to allow for growth of microorganisms on the labeled substrates.

During this incubation period, all bacteria directly or indirectly involved in the utilization of isotope-labeled electron donors will become enriched in isotope-labeled DNA. Following retrieval of the tool from the well, microorganisms may be washed off the surface and their isotope-labeled, higher-density DNA may be separated from background DNA by density-gradient centrifugation. This higher-density DNA (and the non-labeled DNA) can then be analyzed using known molecular techniques. Oligonucleotide microarrys may serve to identify/enumerate target-specific organisms whereas clone libraries may be used to identify novel, uncultured microorganisms. The device may be used in conjunction with commercially available robotics for automated extraction of DNA. The extent of microbially induced corrosion of metals/radionucliudes may be measured optically by scanning the metal surface with a laser; alternatively, contaminant biotransformation may be detected biochemically via addition of a dye/reporter or electrochemically via measurement of electrical resistance. Analysis of the uranium-coated surface will allow for determining the extent of uranium reduction and the calculation of pollutant removal rates occurring under *in situ* conditions.

Test wells of the device also may be equipped with a matrix allowing for the slow, continuous release of chemicals (*e.g.*, external carbon and energy sources; other nutrients; conditioning agents such as pH or redox agents). The matrix may be a polymer or a membrane vesicle containing the nutrient in question. Diffusion characteristics of the matrix/membrane may be selected to achieve different nutrient levels in each of the test wells if desired. Presented nutrients may be added in solid, liquid or gaseous state. Energy sources may be presented in the presence and absence of pollutant coating.

Some of the test wells may be configured for continuous flow-through operation *in situ*. Flow through the device may be passive or active. In active devices, a small pump would facilitate groundwater movement whereas tubing of various length and configuration may be used to prevent the effluent of one test well to become the influent of another. These test systems will report on intrinsic (bioremediation) biocorrosion potential and rates. Computational analysis of the up to 96 resultant data sets using subtractive profiling will ad a hitherto unattained discriminatory power to the analysis of both microbial community composition and function in subsurface environments (Figure 1).

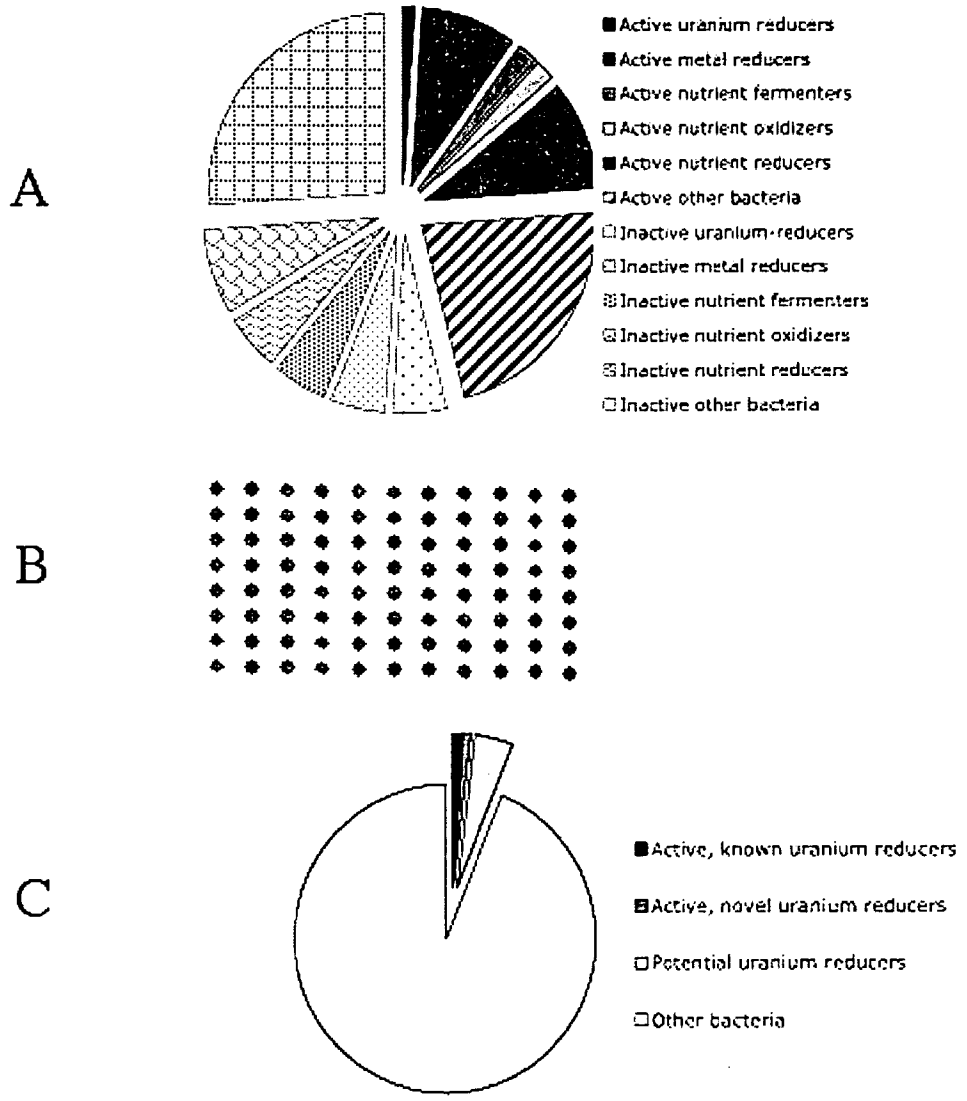


Figure 1.

The above schematic illustrates the utility of the proposed device. Conventional microbial community analysis produces a picture as shown in A. The use of isotope-labeled nutrients can reveal which of the detected microorganisms are alive and active (right half of the community shown in A). Use of the proposed device will allow for the determination of up to 96 community profiles determined under various environmental conditions (B). Computational analysis of the resulting data using subtractive community profiling allows one to identify important pollutant-transforming microorganisms within the large group of active microorganisms (not all metabolically active bacteria are partaking in the bioremediation process). In addition, environmental conditions in the device may allow for the selective enrichment of pollutant-degrading bacteria; some of these may be detected for the first time (C). In addition to the microbial profiling data, optical/chemical analysis of the proposed device will provide data on the rate and extent of biotransformation under 96 different scenarios including the conditions prevailing at the site. This information is critical for designing bioremediation strategies for site cleanup.

The technology is deemed *enabled* in the legal sense as it uses various proven techniques and technologies in a *novel* and *non-obvious* way to achieve the desired goal: the rapid automated analysis of field samples for microbial community composition, degradative potential, and degradative activity under prevalent conditions and under those conditions that may be created *in situ* to accelerate the bioremediation process. Techniques/technologies incorporated in the novel device include:

1) Down-well tools for sampling for monitoring wells
2) Multi-titer-plate testing and fully automated analysis
3) Slow-release compounds for continuous release of microbial nutrients
4) Membrane technology for delivery of nutrients
5) Micro fluidics
6) Laser detection of microbially-induced corrosion
7) Automated DNA extraction
8) Isotope labeling of microorganisms
9) Density gradient analysis for separation of high-density labeled DNA
10) Microbial community analysis using microarrays and bioinformatics
11) Subtractive community profiling for identification of relevant microorganisms.

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Production and Testing of an In Situ Microcosm Array Prototype

Rolf U. Halden, Ph.D., P.E., Assistant Professor of Environmental Health Sciences

Abstract

In situ microcosm arrays (ISMAs) are field deployable devices designed to assist in any of the following tasks: (1) design and monitoring of bioremediation, (2) environmental risk assessment for genetically modified microorganisms and non-native, exotic species, and (3) the discovery of novel microorganisms, enzymes and natural products of interest to the biotech and pharmaceutical industry. In the past six months, JHU has filed three provisional patent applications detailing ISMA sampler design and laboratory analysis strategies. Accordingly, the integrated ISMA technology is a three-step process consisting of the following: (1) deployment of remotely-controlled, submersible, miniaturized test systems (microcosm arrays) that facilitate massive, parallel, biochemical testing of natural microbial communities in situ on a micro-scale, (2) automated, robotic analysis of the sampling devices following retrieval from the target environment (*e.g.,* groundwater monitoring wells, lakes, rivers, and extreme environments such as hot springs, deep sea vents (black smokers) and aquatic biodiversity hotspots), and (3) computerized data analysis, data normalization, and calculation of biochemical turnover rates. A recently completed prior art search suggests that the proposed technology is novel, enabled and non-obvious. A commercialization study is currently underway; initial results identify potential licensees/customers in two market sectors (bioremediation and pharmaceutical industry), each totaling >>$100M per annum. Following review of an ISMA research proposal (R21), the NIH review panel unanimously concluded in their summary statement: "The proposed techniques offer huge promise for improving bioremediation efforts." However, the agency would like to see more preliminary data before committing to the project. Some concerns raised by the review panel over the genomic and proteomic analysis of ISMA samplers have already been addressed in experiments conducted after submission of the NIH R21 research grant. Other concerns relating to the feasibility of in situ microbial enrichment may be overcome best by producing and testing of an ISMA prototype. Overall, significant progress has been made in developing the ISMA technology concept; the prior art search and commercialization study suggest that the technology has broad applicability and excellent potential for generating revenues. The JHU Enterprise Development Organization staff shares this opinion and continues to support the project. Because the work has progressed much more rapidly than anticipated, available funds of the initial TT Seed Grant have been depleted now. The richness of the technology and its revenue potential justify the grant renewal requested with this application. New funds in the amount of $25K will be used (1) to produce an ISMA prototype according to the computer-assisted design (CAD) specifications contained in the provisional patent applications, and (2) to collect a set of performance data at a California Superfund site. Experimental validation of the ISMA sampler needs to be completed by the March 2004 deadline in order to ensure successful intellectual property protection and the broadest possible patent claims.

Introduction

In situ microcosm arrays (ISMAs) are part of an innovative technology designed to facilitate cost-effective and efficient environmental monitoring, environmental risk assessment and biological prospecting (bioprospecting) for novel microorganisms, enzymes and natural products. The integrated ISMA technology consists of three components that are utilized in sequence: (1) deployment of remotely-controlled, submersible, miniaturized test systems (microcosm arrays) that facilitate massive, parallel, biochemical testing of natural microbial communities in situ on the microscale, (2) automated, robotic analysis of the sampling device following retrieval from the target environment (*e.g.,* groundwater monitoring wells, lakes, rivers, and extreme environments such as hot springs, deep sea vents (black smokers) and aquatic biodiversity hotspots), and (3) computerized data analysis, data normalization, biochemical rate calculation/prediction.

The ISMA technology was originally conceived for the purpose of bioremediation. However, a recent patent search and market analysis suggest that the bioprospecting component of the technology may be even more important with respect to profitability and generation of revenue streams.

The following general description of the technology is now being furnished on the Johns Hopkins Medicine Licensing webpage (http://www.hopkinsmedicine.org/lbd/otl/4207.html): *For the management of contaminated sites, the risk assessment of microorganisms introduced into natural environments, and the search for novel microorganisms/enzymes/compounds applicable to biotechnology, a monitoring tool and analysis strategy are disclosed allowing for the automated, rapid and simultaneous determination of the following parameters: (1) water quality and toxicity, (2) intrinsic bioremediation potential, (3) accelerated bioremediation potential following nutrient amendment, (4) effective bioaugmentation strategies for environmental cleanup, (5) turnover rates of natural compounds and environmental pollutants under natural and enhanced conditions, (6) in situ DNA synthesis and protein expression, (7) in situ growth/death rates and metabolic activity of native and introduced biological agents under natural and altered*

environmental conditions, (8) structure and dynamics of microbial communities indigenous to natural soil and water environments, (9) identity and activity of microorganisms of potential value for use in biotechnology.

Potential commercial uses are identified as follows: *The environmental monitoring tool and strategy could be sold as a license, product and/or service. The technology can be used to obtain in a one-step process a comprehensive assessment of contaminated waste sites based on which treatment strategies can be selected, implemented and then monitored, again using the new technology. The invention may be applied to assess the potential risk resulting from the release of pathogens and genetically engineered microorganisms into natural environments. In addition, it has potential value for discovering microorganisms, enzymes and natural products of relevance for the pharmaceutical industry and the biotechnology sector.*

Progress Report

Funding for this project began six months ago on March 1, 2003. In this relatively short period of time, the following work relating to the invention has been completed:

1. In collaboration with the JHU Instrumentation Design Group (IDG), computer-assisted design (CAD) drawings and specifications were generated as shown in Figure 1-3.
2. Laboratory data were generated detailing the proteomic detection of microorganisms and specific functional gene products in complex mixtures of thousands of proteins (Figures 4 and 5). Figure 4 illustrates the use of MALDI MS peptide fingerprinting for the identification of a specific microorganism of interest in the field of bioremediation: strain *Sphingomonas wittichii* RW1, the only bacterium capable of utilizing dibenzo-*p*-dioxin as the sole source of energy and carbon. Figure 5 shows the use of MALDI tandem mass spectrometry for the identification of specific enzymes in complex mixtures containing thousands of proteins. In this case, sequencing of the mass at 3036.34 resulted in the successful detection of the dioxin dioxygenase in crude cell extracts using Mascot database searching. In both instances, results were achieved with minimal sample preparation. These analyses can be fully automated and performed within a matter of minutes. The work illustrates the feasibility of proteomic analysis of ISMA compartments in which specific microorganisms have been enriched using selective nutrient sources. A manuscript describing the methods and results is in preparation (Halden and Wisniewski 2003).
3. Additional laboratory work concentrated on the molecular-genetic characterization of natural microbial communities. Results of these studies have been presented at this year's Annual Meeting of the American Society for Microbiology in Washington, DC (Franklin, Madrid et al. 2003; Xie, Palmateer Oxenberg et al. 2003). These data will serve as a measure of comparison for results to be obtained with the ISMA prototype at the study location. Due to space limitations, the complex phylogenetic trees obtained in these studies cannot be reproduced here. Instead, the concept of stable isotope probing (SIP) is being presented as a strategy for separating ^{13}C-enriched DNA from non-labeled DNA (Figure 6). This methodology can be exploited to separate DNA and identify microorganisms that have taken up specific isotope-labeled compounds contained in the ISMA sampler.
4. Two research proposals were submitted to DOE and NIH, with the respective content being protected by three provisional patent applications (JHU Ref. # 4207):
    a. DOE Natural and Accelerated Bioremediation Research (NABIR) Program. Project title: *Automated Identification of Uranium-reducting Bacteria Using Sampling Arrays, Stable Isotope Labeling, and Molecular-genetics and Proteomics.*

    Status: Not funded; resubmission encouraged. Reviewers' comments: Highly innovative approach to the identification of environmental microorganisms. Project lacks preliminary data on the proteomic analysis of the sampling device; some of the proposed methodologies are not well developed yet. Author's comment: This early proposal did not contain the attached CAD drawings and specification. It also lacked preliminary data on proteomics. Significant progress has been made in both cases, as illustrated by the results shown in Figs 1-3 (ISMA design) and Figs 4-5 (proteomic analysis of microorganisms in complex protein mixtures).

    b. NIH Molecular Structure and Function. Project title: Molecular Assessment of VOC Bioremediation Potential.

    Status: Funding decision pending; priority score of 242 suggests a negative decision in this review cycle. Reviewers' comments: "The proposed techniques offer huge promise for improving bioremediation efforts. Reviewers unanimously approved the innovative aspects of this proposal; however, the panel expressed concern over the microbiological details." Author's comment: Some of the criticism voiced can be addressed using the new data generated after submission of the proposal. Overall, there was a lot of enthusiasm for the study in general and the innovative aspects of the proposal in particular.

5. Frequent meetings and interactions took place with JHU personnel and contractors to explore the commercialization of the ISMA technology; individuals involved include Deborah Alper and Renae Speck (JHU Licensing and Technology Development Office), Nora Zietz (JHU Enterprise Development Organization), Mark Heffernan (Commercialization consultant), and Ruth E. Tyler-Cross (Contracted registered patent agent).

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Halden, R. U., R. N. Cole, et al. (2003). Rapid Detection of Norwalk Virus-like Particles by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry. Exploring the Proteome II, Bethesda, MD, National Institute of Health.

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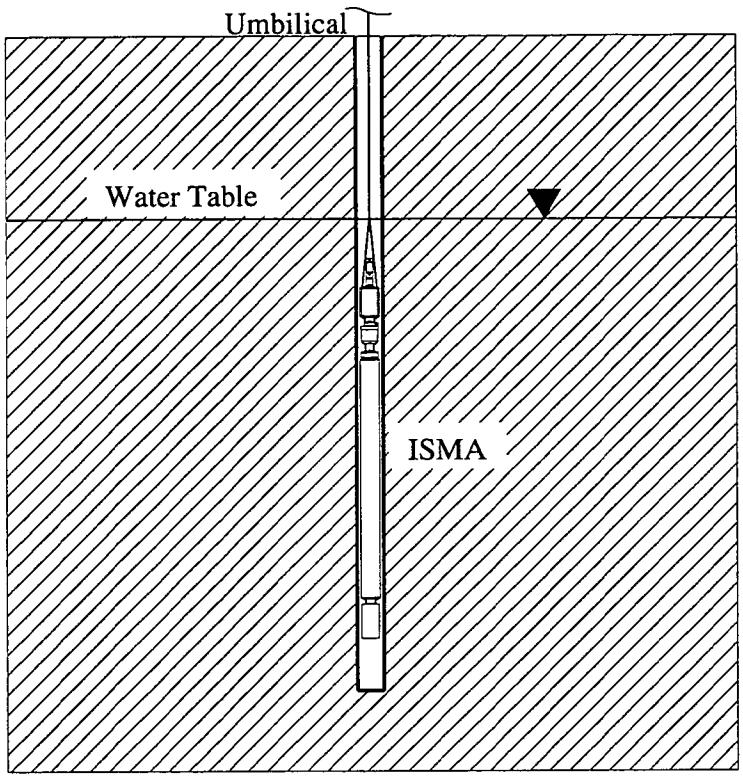
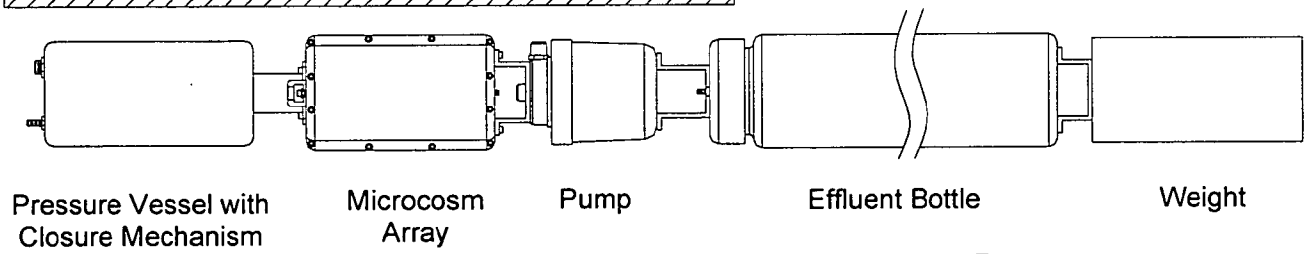
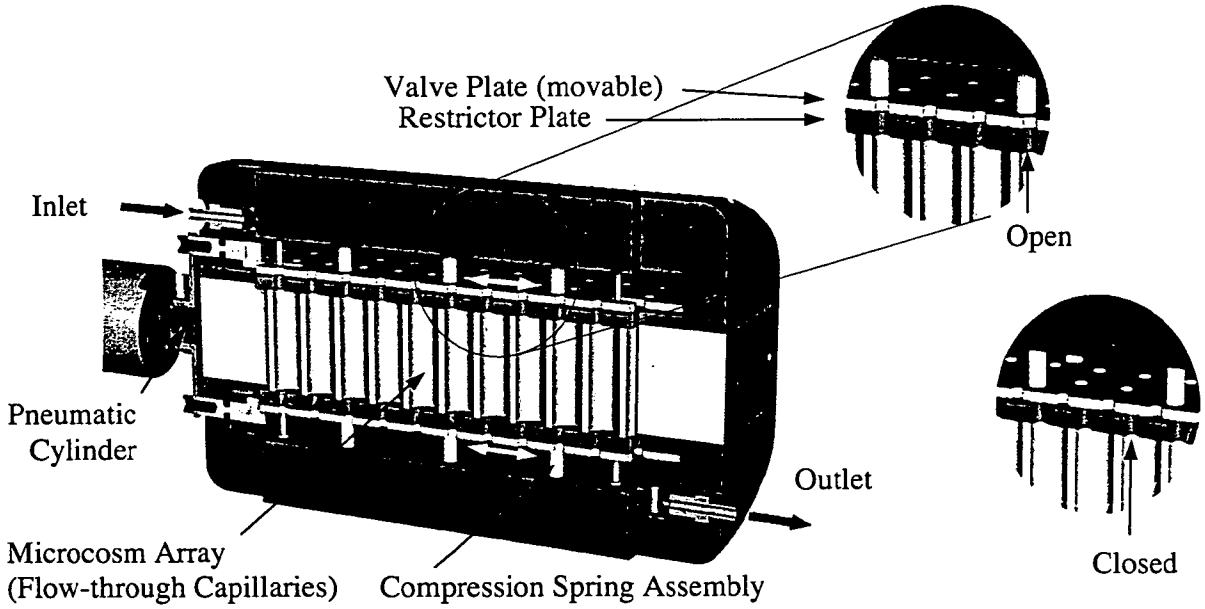


Figure 1.
Schematic showing the *in situ* microcosm array (ISMA) suspended in a standard 100-mm diameter groundwater monitoring well. The device is supported from the surface via an umbilical, that holds it in place and provides power and vacuum for actuation of the integrated closure mechanism and pump.

Figure 2.
In situ microcosm array (ISMA) system components.





Figur 3. Cutaway view of the closure mechanism and the microcosm array (please refer to the three provisional patent applications for additional details.)

Figure 4. Representative mass spectrum of 10^8 typsin-digested cells of the dioxin-degrading bacterium *Sphingomonas wittichii* RW1. The inset spectrum is deisotoped to illustrate the monoisotopic masses submitted to the Mascot database. The mass at 3036 *m/z* corresponds to a prominent dioxin dioxygenase peptide. The peak at 842 *m/z* is a porcine trypsin autolysis product and was used, in part, for internal calibration.

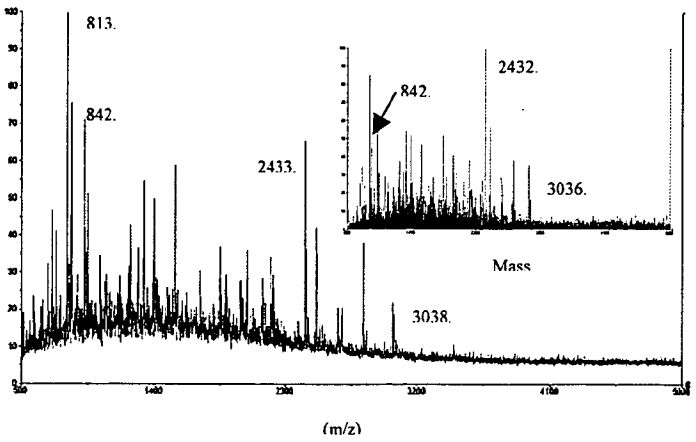
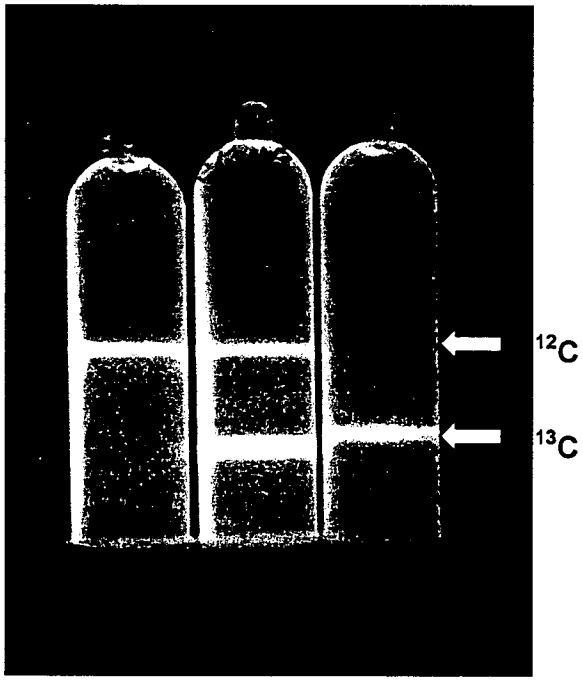


Figure 5. Fragmentation spectrum for the mass at 3036 Da. The inserted table indicates the fragments detected. The ion cutoff value for Sequest-database searching limited the masses used and only 15 of the highlighted 28 masses were used. B ions are generated when the charge is retained on the *N*-terminal side of the fragment while Y ions are generated when the charge remains with the C-terminal side. These data illustrate our ability to detect an enzyme of importance to bioremediation with minimal sample cleanup in a complex mixture of thousands of proteins of one or more microorganims. This type of analysis can be automated and performed within minutes.

A. Acid	B Ions	Y Ions
N	115.05	-
M	246.09	2922.28
N	360.13	2791.24
D	475.16	2677.20
G	532.18	2562.17
D	647.21	2505.15
A	718.25	2390.12
A	789.28	2319.08
M	920.32	2248.05
L	1033.41	2117.01
Q	1161.47	2003.92
Q	1289.53	1875.86
F	1436.59	1747.80
P	1533.65	1600.74
P	1630.70	1503.68
H	1767.76	1406.63
P	1864.81	1269.57
A	1935.85	1172.52
P	2030.90	1101.48
E	2161.94	1004.43
Y	2325.01	875.39
Y	2488.07	712.32
Y	2651.13	549.26
G	2708.15	386.20
P	2805.21	329.18
G	2862.23	232.12
R	-	175.10



Extraction of DNA from soil (Zhou et al., (1996) Appl. Environ. Microbiol. 62:316-322

⇩

Separation of ^{13}C-labeled DNA from ^{12}C-labeled DNA by CsCl high-speed centrifugation

⇩

PCR/ cloning/ DGGE/ sequencing (16S rDNA)

⇩

Phylogenetic analysis

Natural abundance of carbon species: ^{12}C, 98.9%; ^{13}C, 1.1%; ^{14}C isotopes, trace

Figure 6. This Figure is taken from Radajewski et al. (2000) and illustrates the use of stable isotope probing for separating DNA from microorganisms grown on specific ^{13}C-labeled compounds of interest.

Exploring the uncharted microbial world using microcosm arrays

Rolf U. Halden, PhD, PE, Johns Hopkins Bloomberg School of Public Health

Abstract

More than 99% of environmental microorganisms neither grow nor function in laboratory conditions. The present study addresses this challenge by introducing a new research tool for exploring microbial processes in saturated media that harbor a large fraction of the uncharted microbial world. The underlying rationale of this study is that if the microorganisms do not tolerate the transfer from their natural habitat to the laboratory, then the laboratory has to be delivered to the microorganisms. The proposed technology can be broken down into three principal steps: (1) deployment of remotely-controlled, submersible, miniaturized test systems (microcosm arrays) that facilitate massive, parallel, biochemical testing of microbial communities in their natural habitat on a micro-scale, (2) automated, robotic analysis of the sampling devices following incubation in the target environment, and (3) computerized data analysis using genomics/proteomics database searching. The usefulness of this new research strategy will be assessed in laboratory and field experiments concentrating on the biological cleanup of subsurface environments. Specific aims of the proposal are to demonstrate that microcosm arrays (a) can serve to actively capture, concentrate and selectively enrich microorganisms of interest, and (b) that the device can be analyzed using proteomic tools, *i.e.*, mass spectrometric techniques for the rapid analysis of proteins that potentially can reveal both the identity of microorganisms (genotype) and the molecular composition of cells (phenotype). Field-testing of the microcosm array technology will be conducted at a highly contaminated Superfund site that has been characterized previously with state-of-the-art research tools. The new technology potentially will benefit both human and environmental health by improving bioremediation design and by accelerating the discovery of novel microorganisms, enzymes and metabolic processes.

Exploring the uncharted microbial world using microcosm arrays

Rolf U. Halden, PhD, PE, Johns Hopkins Bloomberg School of Public Health

Introduction. In the second half of the 17th century, Antonie van Leeuwenhoek discovered the microbial universe using a primitive microscope. Today—some 350 years later—most of the microscopically observable microorganisms (>99%) remain uncultivated and their individual metabolisms obscure. This proposal describes a new research strategy that employs *in situ* microcosm arrays for studying the ecological significance of microbes, including those that refuse to grow and function outside of their natural habitat. The proposed approach may be exploited to selectively enrich specific members of the microbial community and, by use of isotope-labeled substrates, to unambiguously link them to a specific biochemical reaction of interest. Although microcosm arrays potentially have broad applicability, this proof-of-principle study will focus on one particular use: the biological cleanup (bioremediation) of contaminated groundwater.

The technology demonstration site—located at DOE Superfund Site 300, CA—is the highly contaminated Building 834 Area for which I served as the restoration project leader from 1998 to 2001. This location is interesting from a microbiological viewpoint for at least two reasons. First, maximum groundwater concentrations of the primary contaminant, trichloroethene (TCE), have historically been close to the point of saturation (~1,084 mg/L), thereby creating an unusually challenging environment for indigenous microorganisms. Second, TCE was spilled together with tetrakis(2-ethylbutoxy)silane (TKEBS), a silicon-based lubricant that can support both the anaerobic and aerobic breakdown of chloroethenes. Previous laboratory experiments showed that the four branched alkane side-chains of the water-insoluble TKEBS are released under ambient conditions via slow hydrolysis (9). Data from groundwater microcosm studies revealed that the liberated 2-ethylbutanol is fermented to 2-ethylbutyrate, acetate and hydrogen (10). Recently, we provided additional lab and field data demonstrating that the hydrogen generated during TKEBS fermentation is utilized by *Dehalobacter restrictus* and similar microorganisms performing the reductive dechlorination of TCE to *cis*-dichloroethene (DCE) at the site (8, 10). Interestingly, TKEBS can also serve as a growth substrate for aerobic microbes that cooxidize DCE (9). The proposed study will explore the nature of TKEBS-oxidizing microbes that may be responsible for the observed loss of DCE from site groundwater. Due to rainwater infiltration and soil vapor extraction, local groundwater frequently cycles between aerobic and anaerobic conditions; therefore, TKEBS-oxidizing microbes may play an important role in removing the DCE that accumulates as a dead-end product of anaerobic TCE dechlorination.

In order to gain insight into the microbial diversity and phylogeny of this complex subsurface environment, we previously conducted culture-independent microbial community analyses on groundwater from eight monitoring wells located in a chemical gradient ranging from highly-contaminated to pristine (1, 8). These studies made use of DNA extraction, amplification of eubacterial 16S rDNA, denaturing gradient gel electrophoresis (DGGE), DNA sequencing and sequence alignment using ARB software. To date, over 400 DNA sequences have been obtained from the site and analyzed with respect to phylogeny and spatial distribution, thereby providing a detailed picture of the microbial community composition (1, 8). However, these studies also identified the need for ecological research tools that can link detected microorganisms to metabolic activities known to occur *in situ;* specifically, at this point it is uncertain which of the detected microorganisms are partaking in the aerobic turnover of 2-ethylbutanol released during TKEBS hydrolysis. The here proposed microcosm array technique could provide answers to this and similar ecological questions.

Rationale. The underlying rationale of this study is that if the microorganisms cannot survive and/or function outside of their natural habitat, then the laboratory has to be delivered to the microorganisms. The proposed technology can be broken down into three principal steps: (*i*) deployment of remotely-controlled, submersible, miniaturized test systems (microcosm arrays) that facilitate massive, parallel, biochemical testing of microbial communities in their natural habitat on a micro-scale, (*ii*) partially automated, robotic analysis of the sampling devices following incubation in the target environment, and (*iii*) computational data analysis using genomic/proteomic databases and search engines. The usefulness of this research strategy will be assessed in laboratory and field experiments concentrating on the bioremediation of subsurface environments. Specific aims of the proposal are to demonstrate (*A*) that microcosm arrays can serve to actively capture, concentrate and selectively enrich microorganisms of interest, and (*B*) that the use of isotope-labeled test compounds in the device can serve to unambiguously link microbial community function to phylogeny. For this purpose, microcosm arrays will be analyzed with state-of-the-art genomic techniques (*i.e.*, stable isotope probing; SIP (11)) and proteomic tools, *i.e.*, mass spectrometric peptide fingerprinting and peptide sequencing for the rapid determination of proteins that can reveal both the identity of microorganisms (genotype) and the molecular composition of cells (phenotype).

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Potential Importance. The new technology is of great interest to the field of bioremediation as it potentially can reveal in a one-step process the natural composition of microbial communities and their response to the addition of nutrients and microorganisms for accelerated site cleanup (2). Moreover, the selective enrichment of specific microorganisms in individual compartments of the device may function as a virtual "protein amplification reaction," similar to the PCR of DNA: it may increase the concentration of specific proteins (biomarkers) such that conventional time-intensive genomic analyses may be replaced by rapid proteomic techniques for the detection of microbes and enzymes involved in pollutant degradation. Beyond this specific bioremediation application, microcosm array technology potentially may benefit human and environmental health by accelerating the discovery of novel microorganisms, enzymes and microbial processes.

Research Approach. The proposed *in situ* sampling and monitoring tool consists of five major components as shown in Fig.1. The centerpiece is the microcosm array, which is based on the standard 96-well-microtiter-plate format to allow for fully automated analysis using commercially available robotics. Each sampling array holds 96 "capillary microcosms" that can be operated in either batch mode, flow-through mode or a combination of the two. The device is small enough to fit into a standard 100-mm monitoring well, where it can be deployed below the water table at the desired depth. The built-in closure mechanism and the integrated water pump are triggered from the surface via an umbilical tether. Triggering of the device from the surface will cause the two valve plates (Fig. 2) to shift and the pump to start, thereby exposing each of the 96 microcosms to a constant flow of groundwater. Microorganisms suspended in the groundwater will be forced into the capillaries. Each capillary is filled with a filtration material (*e.g.*, inert glass wool plugs or sterilized porous aquifer material). The filtration matrix of some capillaries will be amended with test substances that slowly diffuse from an inert polymer matrix into the groundwater (*e.g.*, agar beads containing microbial carbon and energy sources). Use of isotope labeled test compounds in conjunction with SIP is being proposed to link microbial function to phylogeny ((11) and references therein). The device can be operated in flow-through mode, with deployment durations being dictated by environmental conditions and *in situ* growth rates. Alternatively, after an initial inoculation period in flow-through mode, the closure mechanism can be activated to switch to batch mode, for selectively enriching specific microbial subpopulations and for maximizing the uptake and incorporation of isotopes into biomarkers (e.g., DNA, enzymes). The effluent of the various capillary microcosms is collected in a bladder at the end of the device, with a check valve preventing backflow of liquids. Owing to the presence of the bladder and the unidirectional flow within the device, none of the effluent can escape into the surrounding groundwater. For this reason, the device allows one to test the effect of various environmental manipulations under *in situ* conditions without releasing any of the chemical and biological agents, or impacting in any way the biology, physics or chemistry of the study location.

Proof-of-concept studies using the microcosm array technology will be conducted in the laboratory with defined mixed cultures of partially or fully sequenced microorganisms that serve as surrogates for more complex environmental microbial communities of unknown composition. These experiments will be designed to test the ability of the device to capture and concentrate microbes entering the capillary microcosms in a controlled flow of simulated groundwater. Parameters to be determined in these experiments include the "capture efficiency" for microorganisms suspended in feed water, and the uniformity of loading as a function of capillary position within the device. Initially, a single-pump configuration will be tested. If serious clogging or uneven flow distribution is being observed, a multi-channel pump configuration will be used as described elsewhere (2). Thereafter, uniformly labeled compounds ($^{13}C_7$-benzoic acid; $^{13}C_{12}$-dibenzofuran) will be added to some of the flow-through microcosm compartments in the form of noble agar beads that are mixed into the filtration material (glass wool), and the device will be fed with filtered groundwater containing known quantities of microorganisms capable of utilizing these test chemicals as carbon and energy sources (*e.g.*, testing the growth of the dioxin-degrading strain *Sphingomonas wittichii* RW1 on dibenzofuran; see (4, 6) and references therein). These experiments will provide initial estimates of inoculation periods and operational strategies (sequential use of flow-through and batch modes) suitable for enriching selected microorganisms of interest from complex microbial mixed cultures entering the device. The "capture efficiency" and flow uniformity will be determined by enumerating microorganisms extracted from individual capillary microcosms on selective media as described previously (4-6). Uptake and incorporation of ^{13}C-labeled compounds in laboratory experiments will be determined using proteomic analyses that are faster and less labor-intensive than the use of genomic SIP analysis (11) (see preliminary data for details). Reference mass spectra of non-labeled and uniformly labeled biomarkers will be obtained from cells grown in controlled conditions (*e.g.*, Figs 3 and 4).

Finally, the microcosm array will be deployed at the Building 834 Operable Unit at Site 300, CA, to evaluate its usefulness in field situations and to assess the possibility of linking microbial phylogeny and function. Field tests will be conducted in groundwater monitoring wells for which we already have collected an extensive set of

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chemical and microbial data (e.g., W-834-D3 and -T5) (8-10). In these experiments, non-labeled and ^{13}C-labeled analogs of TKEBS, 2-ethylbutanol, 2-ethylbutyric acid and acetate (10) will serve to identify bacteria involved in the aerobic breakdown of TKEBS, using both proteomic and genomic (SIP) analyses. The biomass collected in these experiments may also be tested for its ability to cooxidize chloroethenes (9).

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11. Wellington, E. M. H., A. Berry, and M. Krsek. 2003. Resolving functional diversity in relation to microbial community structure in soil: exploiting genomics and stable isotope probing. Curr. Opin. Microbiol. 6:295-301.

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Figure 1. In situ microcosm array system components configured for deployment in a monitoring well.

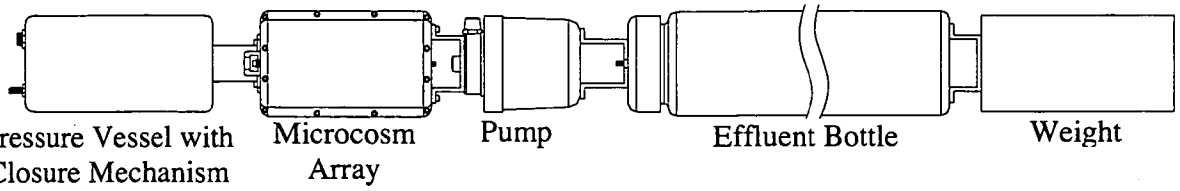


Figure 2. Cutaway view of the microcosm array.

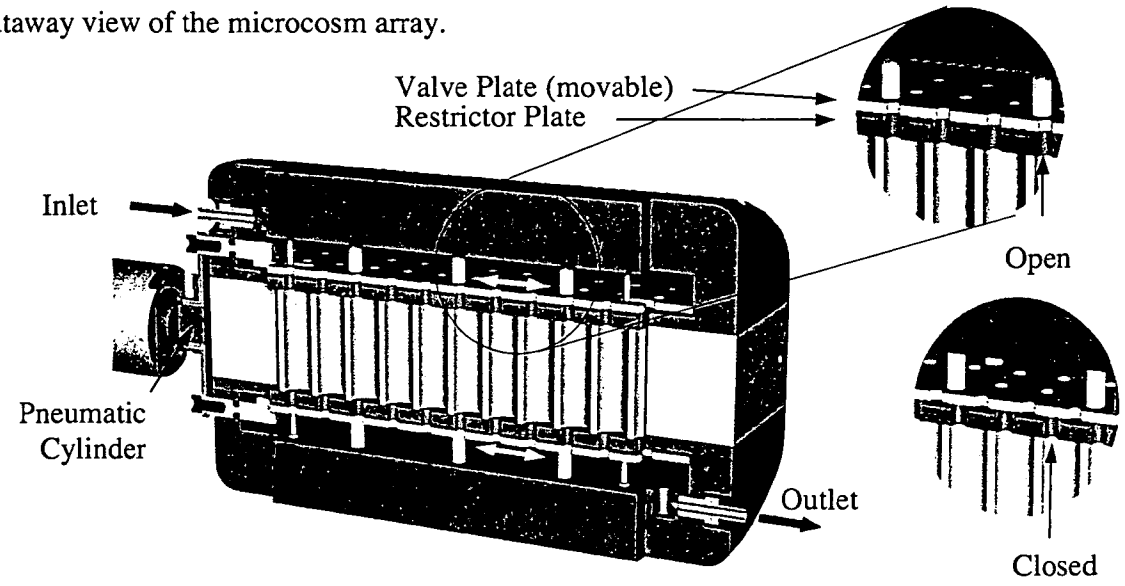
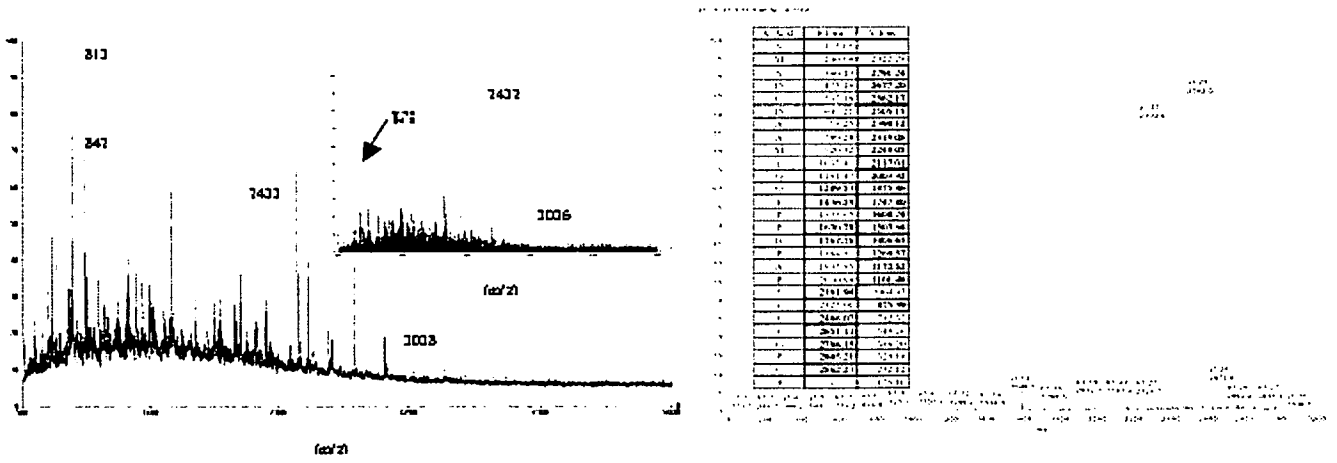


Figure 3. Representative mass spectrum of 10^4 trypsin-digested cells of the dioxin-degrading strain *Sphingomonas wittichii* RW1 (see (4) and references therein). The inset spectrum is deisotoped to illustrate the monoisotopic masses submitted to the Mascot database (score: 72). The peak at 3036 *m/z* corresponds to a prominent dioxin dioxygenase peptide. The peak at 842 *m/z* (arrow) is a porcine trypsin autolysis product and was used, in part, for internal calibration.

Figure 4. Fragmentation spectrum for the peak at 3036 *m/z*. The inserted table indicates the fragments detected. The ion cutoff value for Sequest-database searching limited the masses used, and only 15 of the highlighted 28 masses were submitted. The data illustrate our ability to detect an enzyme of importance to bioremediation with minimal sample cleanup in a complex mixture of thousands of proteins originating from one or more microorganisms. This method is rapid and can be fully automated for high-throughput analysis.



Workable Extent Addendum, dated 12/7/2003

A logical extension of the in situ microcosm array technology is its adaptation for medical purposes, particularly personalized medicine. For this use, the device will be redesigned and significantly reduced in size to reflect a micro-electromechanical system (MEMS). Design types would include rigid and flexible microcosm array assemblies including a small pump or flow equalizer, an inlet for bodily fluids (blood or other), a manifold for distribution of the liquid, a number of compartments that can be operated in flow-through or batch mode, and one or multiple effluent containers for capturing the combined or separate effluents of the various compartments. The compartments can be outfitted with slow-release sorbents/pads from which active ingredients can freely diffuse into the (blood) stream. Alternatively or in addition, the compartments can be outfitted with small quantities of healthy or pathologic cell tissue, e.g., tumor tissue obtained from biopsies. The entire device can be thermally insulated on one side (facing outward away from the patient) and optimized for heat transfer on the other side (skin contact) through selection of appropriate manufacturing materials. The MEMS is connected to the patient's bodily fluid system via an IV or catheter and is strapped or otherwise fastened to the patient's body to reflect as closely as possible the body core temperature. In this way, the device can be used to screen the efficacy of different drugs at various doses in the patient's blood stream without exposing the individual to the compounds tested. In addition, the device can serve to monitor the patient's response to medication and can serve to study the behavior of tumor cells in response to the treatment as well as any adverse reactions of other cell materials that can be included in the device. Analysis of the device can be done by a number of techniques including physical, chemical, and biochemical analyses as well as genomic and proteomic tests as specified previously.

INVENTION DESCRIPTION

Describe the invention completely, using the outline given below.

1. Abstract of the Invention [Briefly describe the invention]

A new tool has been devised for the environmental monitoring of biodiversity and biochemical functions, and for studying the environmental fate of non-native, introduced microorganisms. The tool is made from an inert material and contains a large number of compartments (tens to thousands) designed for capturing microorganisms in their natural environment and for determining their biochemical potential and activities in situ. The compartments of the tool serve as biochemical test vessels. Each compartment contains test substances (organic or inorganic) that may be labeled with isotopes for uptake by or binding to metabolically active microorganisms. The tool is used by exposing the individual test compartments of the device to the matrix of interest (e.g., submerging the device in groundwater) and by incubating it in situ prior to analysis. Typical test compounds are environmental pollutants, electron donor/acceptor compounds, as well as microbial carbon sources and energy sources. The device may also be amended with bacteria/spores/ viruses and protozoa to determine their survival in the environment and to measure any change they may cause, including ecological effects. Following incubation in the test environment, the tool is analyzed for marker compounds (*e.g.*, 16S rDNA, ^{13}C-labeled DNA, ribosomal proteins, etc.) that are characteristic for the trapped indigenous microorganisms. Analysis of isotope-enriched markers allows for identifying metabolically active microorganisms. Additional analyses can be performed on the environmental sample and/or on the inner surfaces of the tool in order to determine biofilm composition and microbially-induced change. These analyses provide both a picture of the microbial community and a corresponding rate of chemical, biochemical and/or physical change. Computational analysis of the multiple community profiles and corresponding rates of change (by e.g., subtractive profiling) can be used to link observed functions to specific microbial community members. This technology is novel in that it combines automated biochemical in situ screening, use of isotopes, in situ sampling and incubation, as well as culture-independent microbial community analysis. It can serve to link observed reactions/activities to distinct members of complex microbial communities. It can be exploited in the environmental monitoring of cleanup sites and in biodiversity prospecting etc. When used to forecast reactions rates in altered environments, systematic biases may occur in the form of "bottle effects" but can be accounted for by standardizing the analysis, and by using algorithms that correct measured rates for biases via interpretation of databases containing pairs of predicted and actual rates measured following environmental manipulations (e.g., nutrient and oxygen addition). The device also may be applied in bioaugmentation studies and in assessing the environmental survival and impact, as well as the risk posed by introduced non-native microorganisms.

2. Problem Solved [Describe the problem solved by this invention]

The new tool and analysis strategy allows one to determine the microbial community structure of complex environmental mixed cultures, as well as to link an observed chemical, biochemical and/or physical change to a particular microorganism. Due to the incubation of the tool in situ, rates determined with the device are expected to closely mirror actual rates currently or potentially occurring *in situ*. The use of isotopes in conjunction with molecular-genetic and/or proteomic analysis techniques allows one to distinguish dead and dormant microorganisms from metabolically active ones (only viable cells will incorporate the label into biomarkers). Parallel testing of effects caused by various environmental parameters (*e.g.*, type and concentration of added nutrients/mixtures/microorganisms) allows one to deduce which of the metabolically active microorganisms are responsible for an observed change. This has important implication for the design and monitoring of bioremediation strategies, e.g. bioimmobilizaiton of uranium by bacteria. Taken together, these characteristics of the new technology provide a hitherto unattained level of discriminatory power that will enable one to selectively enrich for and identify novel microorganisms and microbial functions. This is of great importance for the cleanup (bioremediation) of contaminated sites and for the biological prospecting for novel microorganisms. Furthermore, the technology can be used for assessing the survival and metabolic activity of foreign species in natural environments, which is of importance to public health.

3. Novelty [Identify those elements of the invention that are new when compared to the current state of the art] The tool and analysis strategy are novel because they combine solid-phase sampling techniques, in situ enrichment and biochemical screening, use of electron donor/acceptor pairs and isotope labeling. They are novel in that they provide data for tens or even hundreds of hypothetical environmental scenarios and allow one to determine the likely rates of environmental change induced by these perturbations. The strategy is novel in that it makes use of in situ microcosm arrays in conjunction with culture-independent microbial community analysis to obtain a comprehensive picture of microbial communities and to link specific microbes to observed reactions by using computer-assisted subtractive profiling techniques. The proposed inclusion of miniaturized pumps and closure mechanisms into the in situ microcosm array sampler is new as it will allow one to first inoculate and then incubate the device in the environment without removing (and potentially harming) the resident microbes from their natural environment. The device also allows one to determine how non-native microorganisms will cope in natural environments when confronted with physical, biological and/or chemical stressors. For this application, test organisms will be inoculated into the device prior to its deployment. Semi-permeable membranes can allow the introduced species to come into contact with the target environment while staying contained in the device.

4. Detailed Description of the invention:

On a separate page(s), attach a detailed description of how to make and use the invention. The description must contain sufficient detail so that one skilled in the same discipline could reproduce the invention. Include the following as necessary:

Please refer to the attached two grant proposals and one abstract, taken from a letter of intent sent in response to a request for proposals.

1- data pertaining to the invention;
2- drawings or photographs illustrating the invention;
3- structural formulae if a chemical;
4- procedural steps if a process
5- a description of any prototype or working model;

In general, a manuscript that has been prepared for submission to a journal will satisfy this requirement.

5. **Workable Extent/Scope** [Describe the future course of related work, and possible variations of the present invention in terms of the broadest scope expected to be operable; if a ***compound***, describe substitutions, breadth of substituents, derivatives, salts etc., if ***DNA or other biological material***, describe modifications that are expected to be operable, if a ***machine or device***, describe operational parameters of the device or a component thereof, including alternative structures for performing the various functions of the machine or device]

The proposed technology has a broad workable extent. Microfluidics and closure mechanisms may be integrated into the sampler to separate in time the inoculation of the device (pumps on; closure in open position) from the incubation period that allows chemical change to take place within the sampler's microenvironments (pumps off; device closed). Central facilities may be used for analyzing samplers deployed in situ. This will allow for automated analysis and a for high degree of standardization. Standardized analysis in turn will allow one to determine the systematic biases of the technique (due to "bottle effects"); once identified, these biases can be accounted and corrected for when predicting environmental change caused by engineering interventions. For bioremediation purposes, this would entail the development of databases that record predicted biotransformation rates and rates actually observed in situ. The format of the tool allows for automated analysis. Speed and ease of analysis may be achieved by replacing molecular-genetic analysis with other more convenient measurement techniques suitable for discerning isotope distributions (e.g., use of MALDI TOF mass spectrometry for automated microorganism identification).

The device also may be adapted for studying the fate of either beneficial or hazardous biological agents in natural environments. This work would require the device to be outfitted with a semi-permeable membrane allowing for interaction of the test species with the environment without allowing for its release.

6. **References** [Please list the closest and most relevant journal citations, patents, general knowledge or other public information related to the invention]

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☐ No references available at this time.

Automated Identification of Uranium-reducing Bacteria Using Sampling Arrays, Stable Isotope Labeling and Molecular-Genetics and Proteomics Analyses

1 Abstract

This proposal describes a new strategy allowing for the comprehensive and fully automated assessment of bioremediation potential at sites containing uranium and other contaminants. The proposed robotic-ready *in situ* bioremediation array (ISBA) is a solid-phase sampling device that is deployed via lowering into the monitoring well where, over time, it will be colonized by indigenous microorganisms. Each sampler contains an array of vials or "microenvironments" suitable for the selective enrichment of microorganisms and the determination of their metabolic activities *in situ* under differing environmental conditions. It is hypothesized that: (A) microbial community data obtained with a basic ISBA sampler are comparable to those obtained by the analysis of conventional groundwater samples; (B) integration of electron donors and electron acceptors into the ISBA sampler aids in the identification of uranium-reducing microorganisms, enhances the sensitivity and selectivity of molecular-genetic microbial community profiling, and also provides predictive capabilities, as it conveniently allows for the *in situ* screening of multiple subsurface amendment strategies; (C) integration of stable isotope-labeled electron donor compounds into the ISBA sampler aids in the identification of metabolically active microorganisms; (D) mass spectrometric analysis of ribosomal proteins contained in microorganisms collected on the ISBA sampler can be used to identify microorganisms in environmental mixed cultures; and (E) routine identification of metal-reducing microorganisms can be accomplished using custom-made database software.

The project commences with the development of ISBA prototypes and their evaluation in laboratory microcosms and in the field (FRC, UMTRA, LLNL, APG). The 16S rDNA of microorganisms will be amplified from crude DNA extracts obtained from the individual sampling compartments and analyzed by denaturing gradient gel electrophoresis (DGGE). DNA bands of interest will be sequenced and aligned for phylogenetic analysis using the software package ARB. Microbial community profiles generated with the ISBA sampler will be compared

to those obtained using alternative sampling techniques (*e.g.*, groundwater filtration and other solid-phase samplers). Following validation of the basic ISBA sampling strategy, we will investigate whether electron donor and acceptor compounds integrated in and diffusing from the sampler can be used to selectively enrich uranium-reducing microorganisms that then can be detected more readily. These tests will include the use of carbon-13 labeled acetate. High-density, ^{13}C-labeled DNA of microorganisms that have taken up this carbon and energy source in situ will be separated from non-labeled DNA by cesium-chloride/ethidium-bromide gradient density centrifugation for the purpose of identifying and differentiating metabolically active microorganisms from inactive ones.

Additional studies will explore the use of matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) for the fully automated analysis of ISBA samplers, targeting ribosomal proteins as genus/strain/species-specific biomarkers. This work will commence with the extraction and mass-spectrometric identification of ribosomal proteins from pure cultures. Next, we will analyze defined mixed cultures of metal-reducing bacteria and will modify our existing algorithms and statistical models to account for the signal suppression and the complexity that is likely to be observed in protein mixtures. Then, we will spike known quantities of target microorganisms into uncharacterized environmental samples to determine the sensitivity, selectivity, and robustness of our method. Finally, we will demonstrate the fully automated analysis of a microtiter-plate-based ISBA. For the interpretation of these experiments, molecular-genetic analyses conducted in parallel will serve as benchmarks. Anticipated results of the proposed work include the identification of uranium-reducing microorganisms at field sites, the adaptation and automation of mass-spectrometric techniques for the analysis of microbial mixed cultures, and the launch of a web-based microbial identification database with accompanying freeware.

2 Introduction

Bioremediation is an effective, yet inexpensive biotechnology for removing organic and inorganic pollutants from contaminated environments (14). When targeting dissolved metals and radionuclides, the goal is to convert water-soluble, toxic species to insoluble, less toxic daughter products (23). For example, uranium may be removed from contaminated groundwater and immobilized in the subsurface via the injection of carbon sources that stimulate the microbially induced precipitation of dissolved U(VI) in the form of insoluble U(IV) (23). In this case, the contaminant is being treated "in place" and the process is being referred to as *in situ* bioremediation.

When designing *in situ* bioremediation strategies, it is essential to gain an understanding of the type, activity, and nutritional requirements of subsurface microbial communities present at a specific cleanup site (26). Microbial community information also is important for convincing regulatory agencies and stakeholders that the contaminant is being removed (or, in the case of metals, successfully immobilized in the subsurface) rather than being diluted or dispersed in groundwater.

Currently, the assessment of bioremediation potential at a given site is both labor- and cost-intensive. A typical approach for implementing bioremediation includes the following two steps:

(1) Microcosm screening studies conducted in the laboratory to determine the extent of intrinsic bioremediation and to identify the type, quantity and frequency of carbon source injection that may be needed in order to accelerate the *in situ* bioremediation process. These experiments also serve to estimate contaminant removal rates but do not accurately reflect actual *in situ* removal rates due to the biases introduced by "bottle effects."

(2) Microbial community profiles are obtained from microcosm and field samples to determine the microorganisms responsible for the desired biotransformation reactions. Since most microorganisms fail to grow on laboratory media, culture-independent profiling techniques are commonly used, *e.g.*, 16S rDNA-based analyses.

This proposal describes a new technology that promises to accomplish both of the above tasks in a one-step process, potentially yielding superior results by providing more detailed information of higher accuracy obtained more rapidly and, due to automation, at a potentially lower cost. The fully developed technology will yield information on what types of organisms are present, which are alive and metabolically active, what type of nutrients and nutrient dosages should be used to accelerate bioremediation, and what *in situ* bioremediation rates would result.

2.1 The challenge of detecting and identifying metabolically-active uranium-reducing bacteria

The immobilization of uranium can be performed by a number of microorganisms. Some of these gain energy in the process, some reduce uranium directly in a fortuitous side reaction, and a third group may reduce the radionuclide indirectly via the excretion of waste products, e.g., hydrogen sulfide (see (24) and references therein). Assessing the actual *in situ* uranium-reduction activity of metal-reducing bacteria is challenging, however. Obligate uranium-reducing bacteria representing "reporter organisms" are not known and may not exist. Metal-and sulfate-reducing bacteria having *uranium-reduction potential* are ubiquitous in subsurface environments, due to the great abundance of metals and sulfate in the Earth's crust (23). Therefore, a subset of these should be detectable at all sites supporting microbial life. Thus, probing for metal-reducing bacteria with highly sensitive and selective molecular tools (*e.g.*, the polymerase chain reaction, PCR) has limited value, as it only will confirm the expected presence of some of these organisms in a given location. On the other hand, use of less selective tools (*e.g.*, 16S rRNA gene amplification followed by DGGE) identifies only the most abundant microbial species, and thus most likely will not include potential uranium reducers. Overgrowth by other microorganisms also is expected to occur at sites where carbon and energy sources are introduced into the subsurface to effect anaerobiosis and accelerated uranium reduction. In these situations, fermenting microbes and those utilizing dissolved oxygen, nitrate and other abundant electron acceptors are likely to eclipse the potentially small number of uranium-reducing microorganisms (47).

Another challenge is that the detection of 16S rDNA sequences in environmental samples does not necessarily imply any of the following: (a) the population of the corresponding microorganism is present, (b) it is physically intact, (c) it is viable, (d) it is metabolically active

and (e) it is performing the desired function. Indeed, in field situations it is possible that a specific type of microorganisms can cause a chemical change first observed in a sampling location where neither these microbes nor their DNA is present. This will be the case in heterogeneous subsurface environments where the monitoring well is downstream of the area where the relevant microorganisms reside. Thus, using conventional sampling techniques, spatial factors and limitations in the monitoring network may make it impossible to accurately link an observed chemical change to the responsible microorganism at a given monitoring location.

Finally, groundwater is the preferred sample matrix for profiling of microbial communities (26), as it is both readily available and inexpensive. Unfortunately, the lifestyle of a given target organism has a significant impact on our ability to detect it in this matrix. In the extreme, a target organism pursuing a sessile lifestyle throughout its existence will be impossible to detect in groundwater at a site even if it is present at extremely high densities. Thus, groundwater monitoring alone may not accurately reflect the microbial community composition and dynamics of subsurface environments. Recently, solid-phase samplers were rediscovered as useful tools for overcoming some of these limitations (13).

2.2 Use of solid-phase samplers for biological monitoring

In their simplest configuration, solid-phase samplers are nothing more than a physical surface incubated in an environment of interest for a period of time sufficiently long to allow for the colonization by microorganisms. Buried or submerged glass slides have been used extensively to collect microorganisms from soils. bioreactors and other environments (*e.g.*, (20)).
, reported on the use glass wool as a passive sampling device (13); the material was lowered into groundwater monitoring wells where it passively collected microorganisms over time. Following retrieval of the tool, microorganisms were extracted from the "bug traps" and identified via the detection of biomarkers including DNA, phospholipids, fatty acids and respiratory quinones (13). An argument can be made that microorganisms collected with a solid-phase sampler are more representative of the metabolically active microbial community than those obtained by groundwater sampling because the sampling device requires the active physical attachment by the microorganisms to be captured. However, dead microorganisms, cell debris and DNA also may become entrapped. Highly sensitive tools (*e.g.*, the PCR) can detect biomarkers of in non-living material as well as those of metabolically active microbial community members. Very recently, a novel approach was introduced that exploits stable-isotope markers to distinguish metabolically active microorganisms from those being dormant or non-viable. This promising technique has not yet been used with solid-phase samplers.

2.3 Use of isotope-labeled substrates for linking observed metabolic activities to specific microorganisms

Stable isotope probing (SIP) exploits the fact that the DNA of an organism growing on carbon-13 enriched carbon sources becomes ^{13}C-labeled ("heavier"), thereby enabling one to resolve its DNA from the total community DNA by density gradient centrifugation. The approach was used successfully for the study of methanol-utilizing bacteria in soil (28, 34, 35). The soil of interest was incubated with ^{13}C-labeled methanol, the genomic DNA was extracted and spun down in a

gradient of cesium chloride to separate the "heavy" (^{13}C-labeled) DNA from "light" DNA containing primarily ^{12}C. (A small fraction of ^{13}C also is present in "light DNA" as a result of the natural distribution of this isotope in the biosphere). While representing a powerful research tool, stable isotope probing has limited potential for being applied for routine monitoring at DOE facilities, however; the technique is too time- and labor-intensive. In addition, it may be impossible to automate. An alternative approach for the identification of microorganisms is to look for gene expression products (*i.e.*, proteins) rather than for their characteristic DNA sequences. This can be done with the latest generation of mass-spectrometry instrumentation that offers sufficient speed and sensitivity, while also allowing for complete automation of the analysis process.

2.4 The role of mass spectrometry for microbial identification

Matrix assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), with its ability to induce desorption of protein biomarkers from intact bacteria, fungi, spores and viruses, is a powerful and rapidly emerging technology for fast, portable and robust microorganism identification (10). Initially developed for biodefense applications, this technology clearly also has great potential for environmental monitoring purposes. MALDI-TOF-MS techniques are very rapid (<5 minutes analysis time per sample), have low sample volume requirements (< 1 mL) and have a generic capability to identify microorganisms. The literature (10) indicates that between 5,000 to 10,000 cells need to be present on the sample holder to achieve successful detection. Two recent reviews elaborate on the strengths of this technology and provide an outlook on future applications (10, 21).

2.5 Automation of analysis

Robotic devices have been integrated with MALDI-TOF instruments to an astonishing degree. The latest generation of commercially available robotics allows for the fully automated sample preparation and analysis, including preparation and imaging of 2D gels, harvesting and digestion of the protein spots, and application of the digests to multi-sample MALDI-TOF targets for analysis (22).

This proposal seeks to integrate the above technologies with the goal of providing an innovative strategy for identifying uranium-reducing bacteria in complex microbial communities present at field sites. In the following section, a selected set of preliminary data is being presented to summarize the progress made so far, and to show that our multi-disciplinary research team is both well positioned and qualified to carry out the proposed work.

3.3 *Microbial community analysis of the uranium-contaminated LLNL Site 300*

we have obtained a comprehensive spatial dataset on the microbial community structure of LLNL Site 300. We have extracted the microbial community DNA from the monitoring wells at LLNL Site 300 using the methods described in section 5 of the research plan. The obtained crude DNA was amplified with the DGGE 16S rDNA primers ((42); see section 5.2) and resolved using DGGE (Figure 2). DNA was excised from DGGE gels, sequenced and the DNA sequences put into in phylogenetic dendrograms created with the ARB package. These community data (Figure 1; (12)) complement and expand our previous work, focusing on 16S rDNA and 16S-23S intergenic spacer region sequences obtained from the Building 834 Operable Unit at Site 300 (26). In the meantime our collaborators at LLNL—the Madrid/Taffet hydrogeology team—have implemented a field test for uranium immobilization at the Pit 7 Complex at Site 300. This location is nearby the previously characterized Building 834 Operable Unit (Figure 3), thereby presenting us with an opportunity to exploit our knowledge of the local microbial community for the proposed study. The paragraph below provides some background information on ongoing work at LLNL and how it will be linked to the work contained in the research plan (section 5).

The LLNL Pit 7 Complex contains three unlined landfills that have released depleted uranium to groundwater. The majority of the uranium contamination occurs in a narrow alluvial channel fill aquifer that is a maximum of 25 ft deep. Maximum dissolved uranium concentrations in alluvial groundwater are about 270 ppb. Sorption of dissolved uranium to hydroxyapatite ($Ca_5(PO_4)_3OH$) has been proposed as a suitable technique for passive *in situ* removal of uranium from groundwater at the site. To test the effectiveness of this technique, during early 2003, LLNL installed about 3.5 tons of cow bone char in 10 fully-penetrating boreholes within the alluvium. The cow bone char was mixed with sand (40:60 ratio by volume) to maintain permeability. LLNL is currently monitoring groundwater chemistry in wells up- and downgradient of the emplacement to define the effects on uranium distribution. We intend to deploy our samplers upstream and downstream of the remediation zone to evaluate the sampling device and to detect possible shifts in the microbial community structure. An overview of the study area is provided in Figure 4.

3.4 *Mass spectrometric identification of microorganisms*

3.4.1 Preliminary results for the detection of intact microorganisms by MALDI TOF MS

In the following, we describe a microorganism identification method based on the detection of ribosomal proteins in mass spectra of intact cells (31). The ribosomal method was proposed and

developed by Dr. Pineda and his team at the Johns Hopkins Applied Physics Laboratory and is based on a proposal by Dr. Plamen Demirev et al. (8) to use proteins in genomic databases as biomarkers. This method motivates the mass spectrometry approach proposed in the research plan below. Experiments conducted by Dr. Pineda's team showed that with an optimal measurement protocol (positive-ion mode & sinapinic acid matrix), a simple model of biomarkers based on ribosomal proteins and N-terminal methionine loss, incorporated into a multiple hypothesis test framework, could be used to readily identify pure samples with 100% accuracy from a small database of microorganisms (18 fully sequenced microorganisms and 38 microorganisms total). No false identifications were found. Moreover, a statistical analysis of the results revealed that a high level of performance could be expected even from databases having as many as 1,000 microorganisms, with no additional false identifications.

Demirev et al. (8) showed that the method was surprisingly robust with respect to sample preparation. In particular, we found that it performed nearly as well, (92-95% correct identification rate) when we induced mass spectral variability by using suboptimal experimental protocols. The two false identifications that occurred with suboptimal protocols, were associated with incompletely sequenced microorganisms. Restricted the database to fully sequenced genomes would eliminated incorrect identifications. Finally, Dr. Pineda has recently developed a new on-line database to support microorganism identification by intact-cell MALDI-TOF and ribosomal protein identification (see (30), and Table 1)

To summarize, by exploiting minimal knowledge of molecular microbiology, we created a surprisingly robust and accurate system for identifying intact cells from mass spectra of intact proteins. The performance of this approach was comparable to identification by mass spectral fingerprint methods.

3.4.2 Preliminary results for the detection of ribosomal biomarkers by MALDI TOF MS

Here we summarize the ribosomal protein identification method developed by Dr. Pineda's team. In previous studies, ribosomal proteins were used as biomarker to identify microorganisms by MALDI TOF MS. The method depends on the observation that ribosomal proteins are highly abundant, fly well in mass spectrometers, and are sufficiently distinct in molecular mass and peptide sequence (Table 2). In *E. coli*, for example approximately 21% of the cell's protein content resides in just fifty-six ribosomal proteins (see Figure 5). Not surprisingly, these proteins are commonly observed in mass spectra of "intact cells" (intact cells are lysed upon addition of matrix). Demirev *et al.* determined that accounting for potential *N*-terminal methinine loss, could improve statistical significance (*p*-values) by an order of magnitude (7). Therefore, to correctly calculate the mass of ribosomal proteins from their sequences, it is necessary to model *N*-terminal methionine loss. This is done by *in silico* cleaving the *N*-terminal methionine in each protein according to probabilities detailed by Hirel (16).

The database used in the study was constructed from the combined SWISSPROT and TrEMBL databases (SWISSPROT Release 39.7 of 02-Oct-2000 and TrEMBL Release 14.17 of 01-Oct-2000). Protein fragments were excluded from consideration, as were sequences that contain

ambiguous residues (*i.e.*, "amino acids" B, X, or Z). The mass of each ribosomal protein was calculated from its appropriately post-translationally modified sequence.

The ribosomal method was validated with a blind study. The database used in the blind study had 38 candidate microorganisms, but only 18 of these had 20 or more ribosomal proteins in the requisite mass range and were thus considered identifiable (Currently Dr. Pineda's database has over 70 identifiable microorganisms).

To identify an unknown microorganism from its mass spectrum, a list of experimentally derived masses was compared to the ribosomal biomarker mass lists of the microorganisms in the database. The significance of identifications was scored with *p*-values based on a model of how false matches between mass spectral peaks and biomarkers are distributed (32). *P*-values represent the likelihood of accidentally getting the observed number of matches between mass spectral peaks and proteins in a proteome. The identification algorithm selects the microorganism with the smallest *p*-value, provided the smallest *p*-value is less than a Bonferroni corrected (17) threshold *p*-value of the form *(1-f)/N* which accounts for the number of microorganisms in the database (*N*), and the desired confidence level (*f*). The algorithm makes no identification if no microorganism in the database has any *p*-values below this threshold.

The blind study sought to identify 5 microorganisms (*B. subtilis, E. coli, P. aeruginosa, H. influenzae* and *B. stearothermophilus*). These microorganisms had 20 or more ribosomal biomarkers in the designated mass range (4 to 13 kDa). To test the identification performance as a function of the number of biomarkers, two microorganisms (*S. typhimurium* and *M. luteus)* with less than 10 biomarkers each were included. One negative control also was included (*A. cloacoa,* a microorganism with no biomarkers in the database). To grow the microorganisms we used standard protocols appropriate for each microorganism were used (31). Although optimal conditions for identification from intact cells have already been established (*e.g.*, positive-ion mode & sinapinic acid) (36) (10), experimental mass spectra were generated under four different conditions to simulate mass spectral variability. In particular, two different acidic matrices (sinapinic acid and α-cyano-4-hydroxycinnamic acid) and two different ionization modes were used. Mass spectral analysis was performed with a Kratos Kompact MALDI Discovery instrument equipped with a 337-nm N_2 laser. Mass spectral peak masses and amplitudes were computed using the bundled Kompact software from Kratos Analytical Ltd. Internal mass calibration was performed using the singly and doubly charged ions of equine cytochrome c. The singly and doubly charged calibrant ions were eliminated from the peak lists. Figure 6 shows typical mass spectra for *B. stearothermophilus* with four different sample preparations. Peaks that match *B. stearothermophilus* ribosomal proteins are indicated with triangles.

Classification results from the blind study are summarized in Table 3. Under optimized conditions, perfect classification was obtained (sinipinic acid and positive-ion mode).

In the robustness tests, positive ion mode yielded the best results with a 98% detection rate vs 92% for negative ion mode, at the 95% confidence level. The bulk of the p-values used for correct identifications range between 10^{-4} and 10^{-12}. These are well separated from the *p*-values associated with incorrect identifications (see Figure 7, below). The best separation is obtained with sinapinic acid in positive-ion mode. Separation of the *p*-values was sufficiently good with

the optimal protocol such that perfect identification could have been achieve even on a database with 1,000 microorganisms (30).

3.4.2.1 Strain identification

The ability to distinguish strains in pure samples, with the intact-cell/intact-protein approach (7), was demonstrated with an early version of the database, using *H. pylori* as a model species. In particular, it was demonstrated that strain 26695 (obtained from ATCC) could be distinguished from strain J99. A subsequent reanalysis with a more recent version of the database improved the ability to distinguish the two strains by two orders of magnitude (as indicate by the *p*-value). The improvement was due to the reduction in false matches that occurred when the database was restricted to ribosomal protein biomarkers.

3.4.3 Rapid identification of microorganisms based on proteolytic digestion

Recently, a team at the University of Maryland demonstrated a novel approach for the rapid identification of viruses (48) (or other organisms having a limited number of potential biomarkers, *e.g.*, spores). Experimentally, a sample of intact virus was digested for a short time (< 5 minutes) with a selective protease, *e.g.*, trypsin. The digestion products were subsequently analyzed by MALDI-TOF mass spectrometry, without fractionation or purification. The bioinformatics strategy generalizes the usual approach of identifying a protein from its signature of proteolytic fragments. In this case, the signature of proteolytic fragments is formed from a mixture of proteins that characterize the microorganism. Proof-of-concept results were obtained by identifying the Sindbis virus from a database containing approximately 5,000 fragments from six viral proteomes. We believe that this same approach can be extended to more complex microorganisms provided the ribosomal protein content of the sample is enriched so as to reduce the density of mass spectral peaks and the corresponding likelihood of false matches.

Given the sum of the data presented in section 3, it is evident that ribosomal biomarkers hold great potential for the identification of microorganisms. Whereas identification of bacteria by mass spectral fingerprints is highly dependent on the culture conditions used to grow the cells, ribosomes make for a better target as they are present in all viable cells at significant concentrations.

Thus, based on our preliminary data we propose five hypotheses to be tested by focusing on the six specific aims listed below.

5 Research Plan

5.1 *Design, assemblage and deployment of ISBA prototypes in microcosms and in the field*

The ISBA prototypes will be based on commercially available 96-position (8x12) deep-well microtiter plates (Wheaton Scientific Products, www.wheatonsci.com/html/Microtiter.html). Each well or "microenvironment" of the sampler will consist of a borosilicate glass vial (1.5 mL; 9x44 mm). Deployed vials either will be empty (for passive collection of bacteria on the glass surface), or they will be filled partially with a matrix (*e.g.*, Noble agar; 0.5-2%) or an alternative material (*e.g.*, microscopic beads) offering a large surface area and facilitating the slow release of test compounds in various combinations and at differing concentrations. Some vials will contain the matrix only to determine its influence on the formation and composition of biofilms. For simplicity, initial experiments will be conducted with individual glass-conical-bottom vials rather than with the whole microtiter plate. This will allow us to deploy the sampler in small-diameter boreholes and it also will prevent/minimize cross-contamination between the different "microenvironments" created by the sampler *in situ* (The potential cross-contamination issue will be addressed separately as discussed later). All prototypes will be deployed in sets of replicates that are spatially separated. This will be done by tying sets of identical test vials to a string and by lowering this apparatus into the monitoring well, with each set being separated from the next by a minimum of six inches in depth; following deployment, the vials will be retrieved by using the string. This deployment strategy for solid-phase samplers has previously been used by our collaborators (13). A list of initial test compounds to be screened with ISBA prototypes is given in Table 4; the list may be expanded based on the particular experiments planned at the field sites during our study. All compounds and mixtures will be tested in a concentration range spanning about five orders of magnitude (*e.g.*, 1, 10, 100, 1000, 10,000 ppm $_{(w/v)}$; see Table 4 for details). All experiments will be carried out in at least three replicates. For individual assays, additional vials may be used and pooled for analysis to increase both the yield of DNA/protein and the sensitivity of the assay. The optimal length of *in situ* incubation time will be determined experimentally.

For off-campus experiments, glass vials and ISBA arrays will be prepared and shipped overnight on "blue ice" to our collaborators for deployment in microcosms and in the field. For regulatory reasons, uranium may not be a viable test compound in field situations. In these instances, the radionuclide will be replaced with iron, whose presence also should foster the enrichment of potential uranium reducers (*e.g.*, (25)). In samplers containing pairs of electron donor and acceptor compounds, we will take into consideration the stoichiometry of the respective biochemical reaction. Research groups that agreed to test the sampling device include the Krumholz/Istok team at the FRC, the White/Long team at UMTRA sites and the Madrid/Taffet team at LLNL Site 300, CA. Additional ISBA prototypes will be deployed in microcosms studies to be conducted with APG sediment by the Bouwer/Ball research team as mentioned earlier. The location of deployment is not really critical when evaluating the ISBA analysis technique. Therefore, we will select and prioritize deployment locations with input from Dave Watson and other DOE staff members;

however, we expect to benefit from our prior previously collected information on the microbial community composition at the various field sites. Potential field sites and literature reporting on their corresponding microbial communities are as follows: FRC (13), UMTRA sites (5), LLNL Site 300 (12, 26) and APG (47). By integrating the ISBA sampler into on-going field and laboratory experiments at these selected DOE facilities, we will be able to compare our results to those obtained by other sampling techniques (see section 5.2 for details).

5.2 Task 2. : Analysis of samples obtained by various sampling techniques from identical locations in order to determine the value of ISBAs for microbial community analysis

Crude DNA will be extracted from the three different sampling scenarios, the "bug trap," groundwater filtrate and the sample array. The DNA extractions will be variations on the alkaline lysis method described by Schauer (38) depending on the individual situation. The 16S rDNA will be amplified from the crude DNA according to Teske (42) and the resultant amplified DNA compared using denaturant gradient gel electrophoresis (DGGE) (37). These same methods will also be implemented to analyze the microbial colonization of the (control) vials containing the available growth surfaces of glass, Noble agar (Difco, MD) and Noble agar plus a carbon source.

This technique has been widely used to determine the genetic diversity of natural microbial communities (37), in this case we are using it to analyze the influence of our sampling methods on the obtained picture of the natural community. Some bands will be extracted from the DGGE analysis for sequencing in order to validate the process (see section 5.4).

In order to minimize experimental bias introduced by the biomarker extraction protocol, we will coordinate the sample processing with our collaborators. For the work with Dr. White's group, we are planning to integrate "Janet" (Y.-J.) Chang (5) into our studies to perform biomarker extractions as a team and analyze extract splits ($^{12}C/^{13}C$ DNA), respectively, by conventional DGGE (5) in Dr. White's laboratory and by a combination of density centrifugation/DGGE analysis for separation of labeled DNA in Dr. Halden's laboratory as described below (see Dr. White's letter of collaboration for details).

5.2.1 DNA extraction

Two ml of lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 sucrose and 50 mM Tris HCl pH 9.0) will be used to suspend the bacteria, this suspension will then be incubated at 37°C for 45 min. Proteinase K 0.2 g/ml plus SDS 1% will be added and suspension incubated at 55°C for 60 min. The lysate will be recovered and extracted twice with equal volumes of phenol-chloroform-iso amyl alcohol (25:24:1, pH 8). Excess phenol will be removed by the addition of an equal volume of chloroform. The aqueous phase will be removed carefully and using isopropanol and sodium acetate the DNA will be precipitated, washed with 70% ethanol and resuspended in 300 µl of sterile distilled water.

5.2.2 PCR conditions

The primer combination GM5f-GC (forward) and 907r (reverse) amplifies a 550 bp fragment of the 16S rRNA. The nucleotide sequence of the forward primer, which is specific for eubacteria

(5'-CCTACGGGAGGCAGCAG-3') contains at its 5' end a 40 base GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3') to stabilize the melting behavior of the DNA fragments. The reverse primer used targets the universal consensus sequence (5'-CCCCTCAATTCCTTTGAGTTT-3'). A "touchdown" PCR (9) will be used, in which the annealing temperature is set at 65°C and decreases by 0.5°C every cycle until a touchdown of 55°C, at which temperature a further 10 cycles are carried out. PCR amplification will be performed in a total volume of 50 µl in a 0.2 ml microfuge tube. Each tube will contain 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 9.0), 2% bovine serum albumin (BSA), 100 pmol each dNTP, 50 pmol of each primer and 1 unit of Redtaq genomic DNA polymerase (Sigma, MI). Template DNA at a concentration of 1 ng will be added to the reaction mix. The PCR machine to be used is a PTC-2000 DNA Engine Peltier Thermal Cycling System, MJ Research, MA. PCR products will be identified on 1% agarose gels stained with ethidium bromide and visualized using a UV transilluminator and a gel documentation system (Alpha Imager 2000 V5.5, Alpha Innotech Corporation,VA). Amplified DNA of the correct size will be reconditioned according to Thompson (43), that is, a low cycle number reamplification of a ten-fold diluted template PCR product will be performed to reduce the potential for formation of heteroduplexes.

5.2.3 DGGE

Fifty µl of reconditioned PCR product will be loaded onto the gel to analyze the mixture of PCR fragments obtained by amplifications of the DNA extracted from the sampling wells. The DGGE analysis will be performed as described by Schafer and Muyzer, (37), with 6% (wt/vol) acrylamide gels (in 0.5 TAE: 20 mM Tris acetate [pH 7.8], 10 mM sodium acetate, 0.5 mM disodium EDTA) containing a linear chemical gradient ranging from 35% to 60 % denaturant. Gels will be poured from 6 % (wt/vol) acrylamide stock solutions (acrylamide-N,N-methylene-bisacrylamide, 37:1) containing 0 and 100 % denaturant (7 M urea and 40 % [vol/vol] formamide, deionized with AG501-X8 mixed-bed resin [Bio-Rad Laboratories, Inc.]). The gels will be run for 18 hrs at 60°C and 100 V. Bands will be visualized by staining. The optimal method for staining DGGE gels is ethidium bromide staining, the gel is stained in 100 ml of 1 x TAE buffer containing 50 µg/ml ethidium bromide. This is gently agitated for 15 min, the solution discarded and replaced with distilled water and left for 10 min. The gels will be visualized with a UV transilluminator and the gel documentation system (Alpha Imager 2000 V5.5, Alpha Innotech Corporation, VA).

5.3 *Task 3. Analysis of chemically-amended ISBAs to determine whether the in situ screening of electron acceptors and donors adds discriminatory/predictive power to microbial community profiling*

The microbial community DNA will be removed from the individual vials or microtiter plates as described in 5.2. The samples will be analyzed and their unique "fingerprints" resolved using PCR and DGGE as set out in section 5.4. Those partial 16s rDNA sequences separated using DGGE will be excised from the gel, sequenced and analyzed according to the methods in section 5.4 to distinguish phylogenetic relationships. The phylogenetic relationships will be interpreted graphically using the ARB software to create dendrograms as laid out in 5.4; *e.g.*, Figure 1).

we will quantify the extent of uranium reduction that occurred in ISBA samplers that were amended with the radionuclide prior to

incubation in microcosms. Measurement of U(VI) and U(IV) will be performed as described elsewhere using selected extractions techniques and kinetic phosphorescence analysis (47).

5.4 Task 4. ( ): Molecular genetic analysis of high-density and low-density fractions of crude DNA extracts to determine the usefulness of isotope-labeling in ISBA monitoring

Stable isotope-labeled and non-labeled crude DNA will be extracted from the environment in the same way as described in section 5.2. The ^{13}C-labeled "heavier" DNA will be resolved from the non-labeled "lighter" DNA using density gradient centrifugation as described by Radajewski (33) using cesium chloride ethidium bromide density gradients. The crude DNA extracted will be analyzed, once again, by amplifying the 16S ribosomal genes and separating them according to DNA sequence using DGGE (see 5.2). This method will give us the microbial community fingerprint for those organisms metabolizing the isotope-labeled substrate. The DNA can then be excised from the gels using sterile pipette tips for sequential phylogenetic analysis. Fractions of acrylamide gel containing the DNA will be incubated in 100 µl of sterile dH20 at 4 C overnight. A 1-µl aliquot of this solution is used for PCR amplification. PCR products are re-run under the same conditions to confirm the purity of the DNA and the PCR product purified with the QIAquick-spin DNA purification system (Qiagen) as per manufacturers instructions. The cleaned PCR product will be subjected to cycle sequencing.

5.4.1 Comparative sequence analysis.

All obtained sequences (>500 bp) will be analyzed using BLAST (1) and added together with most important BLAST hits, to an alignment of about 5,300 homologous bacterial 16S rRNA primary structures (27) by using the aligning tool of the ARB software package (http://mikro.biologie.tu-muenchen.de). Sequences will be checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (27). Potential chimera will be eliminated before the phylogenetic trees are constructed.

5.5 Task 5. Adaptation of mass spectrometry for the analysis of mixed cultures

Ribosomal biomarkers ranging from 4 to 20 kDa can be detected directly from intact cells of microbial cultures (31) or from crude whole cell extracts. The protein profile from crude whole cell extracts of mixed cultures is expected to highly complex and few biomarkers from individual species will be detected. Therefore, the detection of microbes in complex mixed cultures presents a significant challenge that we will address in a phased approach. In previous studies involving complex protein mixtures, we have encountered problems arising from signal suppression when using MALDI TOF MS-based techniques. We have already partially mitigated this problem by targeting a judiciously selected set of ribosomal protein biomarkers. This approach allows us to detect characteristic signals in the microbial cytosol, which—owing to the presence of thousands of proteins—represents a complex mixture in its own right. Nevertheless, we seek to improve the signal-to-noise ratio further in order to reliably detect targets in even more complex mixtures (*e.g.*, biofilm lysate). We will accomplish this goal by enhancing the purification of ribosomal proteins and by fractionating complex samples. In order to obtain a benchmark illustrating the best possible result, we will biochemically extract ribosomal targets from pure cultures and use these extracts as performance standards for judging the success of

developing less stringent but more practical cleanup techniques that lend themselves to automation.

The general strategy for Task 5 is as follows:

(a) For method development and evaluation purposes, we will select up to five pure cultures of relevance to bioremediation, with the prerequisite being that the genome of these bacteria has been sequenced at least partially and that the corresponding data indicate a sufficiently large number of unique ribosomal proteins in each selected strain (10 or more unique biomarkers; (31)). Candidate organisms include *Deinococcus radiodurans* (24 characteristic biomarkers; see Table 2; (4, 19)) and metal-reducing bacteria (*e.g.*, *Geobacter* spp.; (11, 18)) that have been or currently are being sequenced for Dr. Lovley's group.

(b) We will purify the ribosomes of the selected pure cultures and use the extracts as analytical benchmarks for developing a more practical sample preparation procedure.

(c) We will experiment with defined mixed cultures (2-5 members) to determine our ability to detect individual targets present in these mixtures at known densities. This work also will require the modification of Dr. Pineda's database software for the identification of microorganisms.

(d) We will develop a sample preparation technique for microbial mixed cultures that is as simple as possible while still allowing to determine targets in complex mixtures.

(e) Next, we will fortify environmental mixed cultures obtained with the ISBA sampler with known quantities of target organisms and analyze theses samples to explore the sensitivity and robustness of our techniques for sample cleanup, MALDI TOF MS, and statistical analysis.

(f) After having optimized and validated our novel microorganism-identification database software, we will analyze field samples from various sites and compare the results to those obtained with molecular-genetic analysis techniques.

(g) Finally, we will conduct a small-scale demonstration of fully automated ISBA analysis (20-35 of the 96 wells) using our developed protocols. These demonstration will include the analysis of some wells containing define mixed cultures.

5.5.1 Extraction and purification of ribosomes from pure cultures to obtain a performance benchmark

We will enrich for ribosomal protein biomarkers from relatively uninformative proteins in complex samples by extracting ribosomes from cell lysates using standard techniques (40). This strategy was successfully applied earlier for the preparation of mass spectrometry samples (3). Accordingly, microorganisms are harvested by centrifugation, grinded with alumina, extracted to capture soluble components, and washed with a sucrose/salt solution through a sucrose cushion

by ultracentrifugation. Given the high abundance of ribosomal proteins in microbial cells (up to 21% of the cell's protein content; (2)) this step may not be necessary in a practical assay. Nevertheless, we include this work with highly purified targets to determine a benchmark or optimal performance level. Highly purified extracts from selected pure cultures (*Deinococcus radiodurans* [ATCC] and *Geobacter* spp. [Dr. Lovley's group]) will be stored at -80 C and aliquots taken as needed to serve as standards. Using pure cultures and extracts, we will determine the optimal parameters for sample-to-matrix ratio and sample amount on the target to maximize signal strength and reproducibility.

5.5.2 Automated separation of complex protein mixtures from mixed cultures by ion exchange chromatography

The objective of this sub-task is to develop a practical sample preparation technique for automated high-throughput analysis. We will increase the number of proteins detected from crude whole cell extracts by fractionating samples via ion-exchange chromatography and, thus, reduce the number of ion species per fraction competing for charge. Our previous work indicates that MALDI TOF MS produces only about 20 discernable peaks per analysis independent of whether the sample contains hundreds or thousands of individual proteins. By fractionating the sample, a larger number of ribosomal biomarkers will be detected and, therefore, increased confidence in characterizing the microbial population will be achieved.

Proteins will be extracted from mixed populations of microbes with 35% acetonitrile/1% trifluoroacetic acid (TFA) and bound to a strong cation exchanger macrospin column (SMM-SCX, The Nest Group, Inc.). Proteins will be eluted from the macrospin column in at least four ammonium chloride salt steps ranging from 10 mM to 1 M ammonium acetate or ammonium chloride. Fractions will be dried, resuspended in 150 mM sinapinic acid (SA) containing 35% acetonitrile/1% TFA and analyzed by MALDI-TOF MS (Voyager DE-STR, Applied Biosystems, Inc) if necessary, in both negative and positive ion mode to enhance the discriminatory power of the assay. The number and concentrations of salt steps will be optimized to recover the largest number of known ribosomal biomarkers from one species spiked into a mixed microbial population. When necessary, salt fractions will be desalted using C4 Ziptips (Millipore Corp., Milford).

Reverse-phase chromatography represents an alternative to ion exchange chromatography and will be tested using C8 Macrospin columns (SMM-SS08V, The Nest Group, Inc.). Protein extracts will be diluted with 1% TFA and bound to the C8 spin column. After washing with 1%TFA, protein will be eluted from the column in at least four steps ranging from 5 to 50% acetonitrile, dried, resuspended in matrix and analyzed by MALDI-TOF MS. If neither of these methods sufficiently reduces the sample complexity, then both affinity procedures will be performed in series. All of these steps can be automated readily by standard column switching procedures using our Ultimate (LC Packings/Dionex) or Surveyor (Thermofiningan) HPLC systems interfaced with the Probot Micro Fraction Collector (LC Packings/Dionex). The Probot also can spot fractions directly onto a MALDI target plate and apply the matrix.

5.5.3 Strategy for statistical analysis of mass spectral patterns

The pattern recognition algorithm used in the existing database is essentially a single-sided multiple hypothesis test that seeks to test the null hypotheses that each of the microorganism in the database did not generate the experimental mass spectrum. This approach, which tests mutually exclusive events, is not suitable for a setting where multiple microorganisms in the database are likely to be represented in the data. Accordingly, it will be necessary to use a test allowing for the detection of multiple microorganisms in a given sample. This necessitates the construction of a model that accounts for clutter peaks (*i.e.*, peaks not due to the microorganism being tested). Statistically, this means the test must be generalized from a single-sided test to a two-sided test. One such generalization, based on a likelihood ratio test has been applied to detect microorganisms in binary mixtures by a group at PNNL (46). Another approach, based on Bayesian methods is under development by Dr. Pineda's group, and has been used previously to detect phospholipids in MALDI mass spectra (15).

I should be noted here that the use carbon-13 labeled substrates will lead to randomly labeled ribosomal proteins, thereby rendering conventional deconvolution and bioinformatics databases useless; this problem will be circumvented by using only non-labeled samplers for mass spectrometric analysis.

5.5.3.1 Method validation

As mentioned earlier, we will validate our methods with a series of (blind) studies based on: (a) artificial mixtures, (b) complex environmental mixtures spiked with known microorganisms, and (c) environmental mixed cultures (biofilm samples) analyzed by both MALDI TOF MS and molecular-genetic protocols. Similarly, we will include samples composed of defined mixed cultures into the demonstration of fully automated ISBA analysis by MALDI TOF MS.

5.5.3.2 Protein confirmation by tandem mass spectrometry

When using MALDI-TOF MS, microorganism-specific ribosomal biomarkers are identified only based on their intact protein mass (31). The more proteins an undefined sample contains, the more likely it is that the mixture contains proteins having identical molecular mass but different biochemical functions than the targeted ribosomal biomarkers. To validate the presence of specific ribosomal proteins observed in the MALDI-TOF spectra, proteins from column fractions will be digested with trypsin and the resulting peptides will be identified by collision-induced dissociation (CID) using tandem mass spectrometry on our ProteomeX (2D-chromatography system interfaced to an ion trap mass spectrometer) or QSTAR Pulsar instruments (electrospray ionization/quadrupole/time-of-flight (Q-TOF) tandem mass spectrometer).

Tandem mass spectrometry will also be used to identify new ribosomal biomarkers—observed in MALDI-TOF spectra—that have not been assigned previously to a microbial species. Electrospray ionization tandem mass spectrometry (ESI/MS/MS) allows for true protein sequencing and produces data of greater intrinsic value than those obtained by MALDI TOF MS (22). For the protein fraction containing the molecular mass range of interest (4-13 kDa), we can

calculate from (22) that trypsin digestion should yield between 2 and 8 characteristic peptides per protein.

5.6 Task 6. Adaptation of microorganism-identification algorithms contained in the existing microorganism-identification database software to allow for the analysis of mixed cultures.

We will incorporate the protocols and algorithms developed in this study into an existing prototype microorganism identification database (31). The database contains approximately 200 microorganisms, out of which approximately 70 contain 20 or more ribosomal biomarkers. The database is periodically updated from the latest release of the Swiss-Prot/TrEMBL database. The database is stored in a relational database management system (MySQL) with a CGI web interface. Perl scripts are used as middleware to integrate MySQL with the CGI web interface. The database is currently being ported to a web server and should be accessible to the public (at http://www.pinedalab.jhsph.edu) by the time this proposal is under review. This intended database will become a new resource for the bioremediation community.

6 Strengths and Limitations

An important strengths of this study is that the various technologies that are combined in the ISBA sampling/analysis strategy are already mature or at least partially developed: (*a*) solid-phase samplers have been successfully applied for microbial sampling (13); (*b*) high-throughput screening is routinely performed in the biotechnology sector by using miniaturized microtiter plates/assays in various formats (96, 384 or 1536 well plates), (*c*) robotics for the analysis of microtiter plates are standard laboratory equipment; (*d*) slow-release compounds are routinely used in the laboratory and in the field (29, 41), (*e*) stable isotope labeling is a rapidly developing technology (34, 35); (*f*) MALDI TOF MS has been successfully used for the detection of bacteria, viruses and spores (see (10) and references therein); and (*g*) microbial genome sequencing efforts and the development of bioinformatics tools for data mining are rapidly increasing (6). All of the above factors suggest that the proposed project is feasible and likely to advance the biological monitoring capabilities at DOE legacy sites. The project is strengthened further by our access to the latest instrumentation in mass spectrometry. For example, the Applied Biosystems Voyager DE-STR MALDI-TOF-MS has a 20-times greater mass accuracy, a 6-10 times higher resolution and greater sensitivity than the Kratos Kompact instrument used to generate some of the preliminary data we presented. Furthermore, the great variety of instrumentation available to us for sample preparation/analysis and the experience of our research group increase the likelihood of success with this project.

In this project, we will create a tool that can link specific reactions occurring *in situ* to the responsible microorganisms. This will be achieved by combining several techniques, including ^{13}C-labeling, selective enrichment under various environmental conditions, *in situ* screening of multiple pairs of electron donor/acceptor compounds, and the potential for computational analysis of the numerous individual community profiles using subtractive profiling techniques; the process is illustrated in Figure 8.

The development of mass spectrometric techniques for the identification of microorganisms in mixed cultures is another important outcome.

Another important and tangible product of the work is the generation of microorganism-identification database software and search algorithms for interpreting MALDI TOF mass spectra of ribosomal biomarkers and microorganisms. These tools will be updated frequently and will be available to other researchers over the world wide web.

8 Significance

Substantial cost savings may be realized if the mature technology is successfully transferred to the field. Potential areas of application include: (a) initial site assessment, (b) bioremediation design studies, (c) monitoring of accelerated *in situ* bioremediation, and (d) post-treatment and routine monitoring. Savings will result from the automated large-volume high-throughput analysis, and from the elimination of numerous field sampling activities, microcosm experiments and customized microbial analyses. Thus, the developed bioremediation assessment tool will support the environmental restoration and long-term stewardship of sites containing radionuclides and other contaminants.

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Species	Biomarkers
Aeropyrum pernix	63
Anabaena sp .	24
Aquifex aeolicus	53
Archaeoglobus fulgidus	59
Bacillus halodurans	47
Bacillus stearothermophilus	45
Bacillus subtilis	64
Bacillus subtilis	58
Borrelia burgdorferi	51
Brucella melitensis	23
Buchnera aphidicola	72
Campylobacter jejuni	26
Caulobacter crescentus	23
Chlamydia muridarum	53
Chlamydia pneumoniae	53
Chlamydia trachomatis	53
Clostridium acetobutylicum	24
Clostridium perfringens	23
Deinococcus radiodurans	24
Escherichia coli	59
Escherichia coli O157:H7	55
Haemophilus influenzae	53
Haloarcula marismortui	50
Halobacterium sp .	20
Helicobacter pylori	53
Helicobacter pylori J99	38
Lactococcus lactis	32
Leptospira interrogans	25
Listeria innocua	26
Listeria monocytogenes	26
M. thermoautotrophicum	59
Methanococcus jannaschii	62
Methanococcus vannielii	24
Methanopyrus kandleri	23
Mycobacterium leprae	52
Mycobacterium tuberculosis	57
Mycoplasma capricolum	22
Mycoplasma gallisepticum	23
Mycoplasma genitalium	52
Mycoplasma pneumoniae	52
Mycoplasma pulmonis	23
Neisseria meningitidis	31
Pasteurella multocida	29
Pseudomonas aeruginosa	28
Pyrobaculum aerophilum	26
Pyrococcus abyssi	57
Pyrococcus furiosus	27
Pyrococcus horikoshii	58
Ralstonia solanacearum	23
Rhizobium loti	23
Rhizobium meliloti	26
Rickettsia conorii	27
Rickettsia prowazekii	54
Salmonella typhi	34
Salmonella typhimurium	37
Staphylococcus aureus	36
Streptococcus pneumoniae	32
Streptococcus pyogenes	45
Streptomyces coelicolor	46
Sulfolobus acidocaldarius	29
Sulfolobus solfataricus	53
Sulfolobus tokodaii	28
Synechococcus sp .	28
Synechocystis sp .	53
Thermoanaerobacter tengcongensis	20
Thermoplasma acidophilum	30
Thermoplasma volcanium	27
Thermotoga maritima	52
Thermus thermophilus	37
Treponema pallidum	49
Ureaplasma parvum	25
Vibrio cholerae	28
Xylella fastidiosa	24
Yersinia pestis	27

Table 1.
Currently, the database contains 250+ microorganisms. A selection of these having 20 or more biomarkers are listed here.

Table 2. Example of proteins listed by bacterium in the database (Source: Pineda).

Deinococcus radiodurans (vegetative)

embl ID	mass	post-translational modification	description
RL36_DEIRA	4309.22		'50S ribosomal protein L36'
RL34_DEIRA	5608.5		'50S ribosomal protein L34'
RL33_DEIRA	6228.26	Met-loss	'50S ribosomal protein L33'
RL32_DEIRA	6660.74	Met-loss	'50S ribosomal protein L32'
RL35_DEIRA	7294.83	Met-loss	'50S ribosomal protein L35'
RL29_DEIRA	7759.95		'50S ribosomal protein L29'
RL31_DEIRA	8581.81		'50S ribosomal protein L31'
RL28_DEIRA	8831.34	Met-loss	'50S ribosomal protein L28'
RL27_DEIRA	9458.86	Met-loss	'50S ribosomal protein L27'
RS16_DEIRA	9667.04		'30S ribosomal protein S16'
RS20_DEIRA	9871.49	Met-loss	'30S ribosomal protein S20'
RS14_DEIRA	10076.6	Met-loss	'30S ribosomal protein S14'
RS18_DEIRA	10492.3	Met-loss	'30S ribosomal protein S18'
RS19_DEIRA	10691.4	Met-loss	'30S ribosomal protein S19'
RS6_DEIRA	11672.2		'30S ribosomal protein S6'
RS10_DEIRA	12109.1		'30S ribosomal protein S10'
RL7_DEIRA	12496.3	Met-loss	'50S ribosomal protein L7/L12'
RL20_DEIRA	13826.0	Met-loss	'50S ribosomal protein L20'
RS12_DEIRA	14222.7	Met-loss	'30S ribosomal protein S12'
RS9_DEIRA	14601.9	Met-loss	'30S ribosomal protein S9'
RL9_DEIRA	16065.5		'50S ribosomal protein L9'
RL10_DEIRA	17622.8	Met-loss	'50S ribosomal protein L10'
RS7_DEIRA	17810.5	Met-loss	'30S ribosomal protein S7'
RL19_DEIRA	18315.8		'50S ribosomal protein L19'

Met-loss by automatic rule.

Hard Targets	annotated ribo-proteins	replicates per trial	Positive ion mode		Negative ion mode	
			CHCA	SA	CHCA	SA
			Trial 1	Trial 2	Trail 3	Trial 4
Bacillus subtilis	31	5	100%	100%	100%	100%
Escherichia coli	30	5	100%	100%	100%	100%
Pseudomonas aeruginosa	26	5	100%	100%	100%	60%
Haemophilus influenzae	25	5	100%	100%	80%	100%
Bacillus stearothermophilus	20	5	80%	100%	80%	100%

Table 3. Influence of MALDI analysis conditions on the accuracy of target microorganism identification (Pineda et al. 2003).

Compound	Phenomenon studied
None	Biofilm formation on the glass surface
None (plus agar)	Effect of basic matrix (*e.g.*, Noble agar) on community composition
Acetate	Effect of carbon source on community composition
$^{13}C_2$-Acetate	Usefulness of isotope-labeling for identifying metabolically active microorganisms (molecular-genetic approach)
Uranium (VI)* or Fe(III)	Effect of U(VI) on community composition
Acetate + U(VI)* or Fe(III)	Effect of U(VI) plus acetate on community composition
$^{13}C_2$-Acetate + U(VI)*/Fe(III)	Enrichment of uranium- and/or metal-reducing bacteria

* For regulatory reasons, uranium may have to be replaced with Fe(III) in field situations; concentrations of metals (solids) will be approximate only.

Table 4. Matrix of experiments to be conducted with the ISBA samplers (microcosm and field studies).

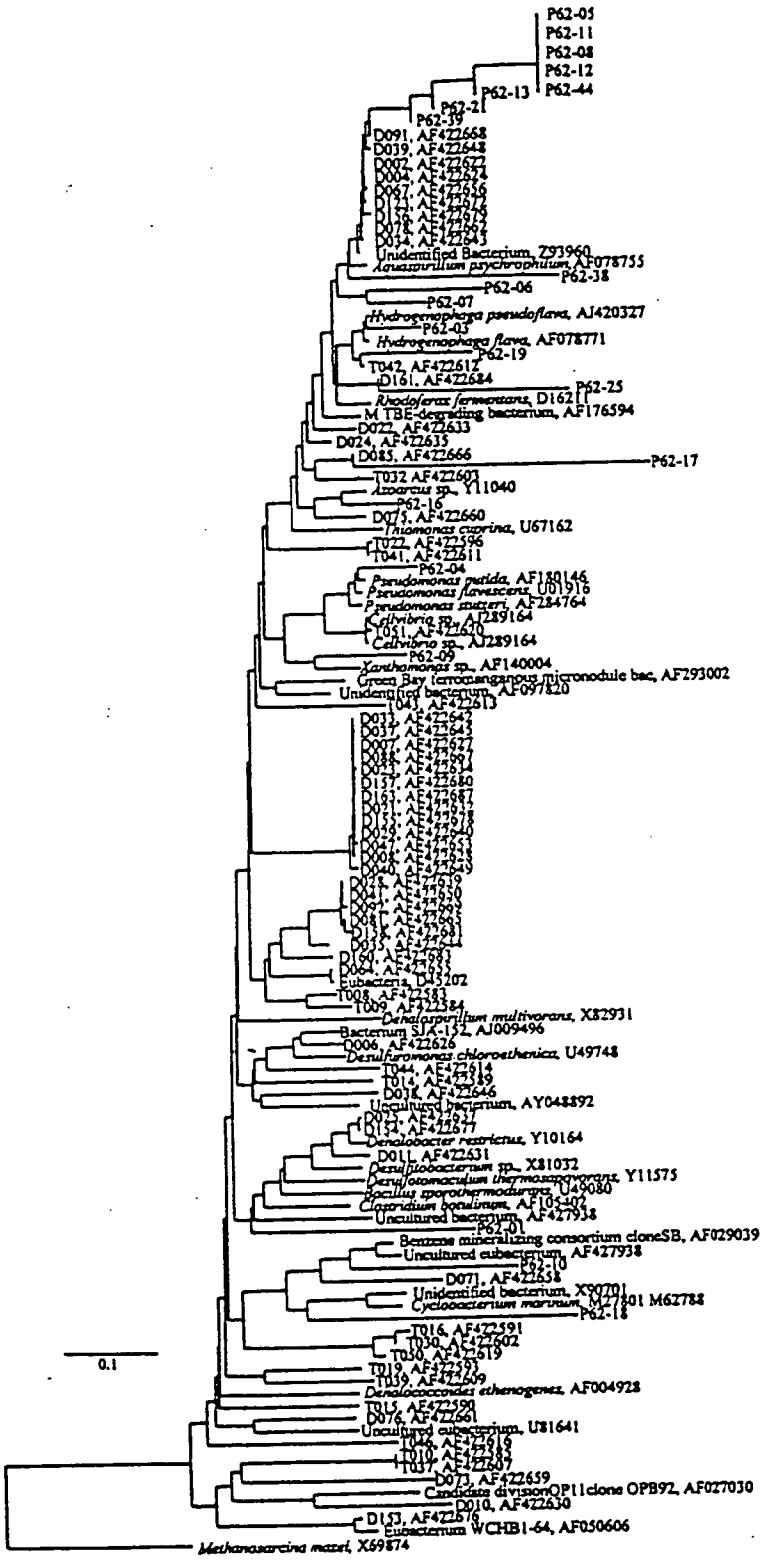


Figure 1. Dendrogram showing published 16S rRNA gene sequences with those obtained from various monitoring wells within the Building 834 Study Area (taken from Franklin et al. 2003).

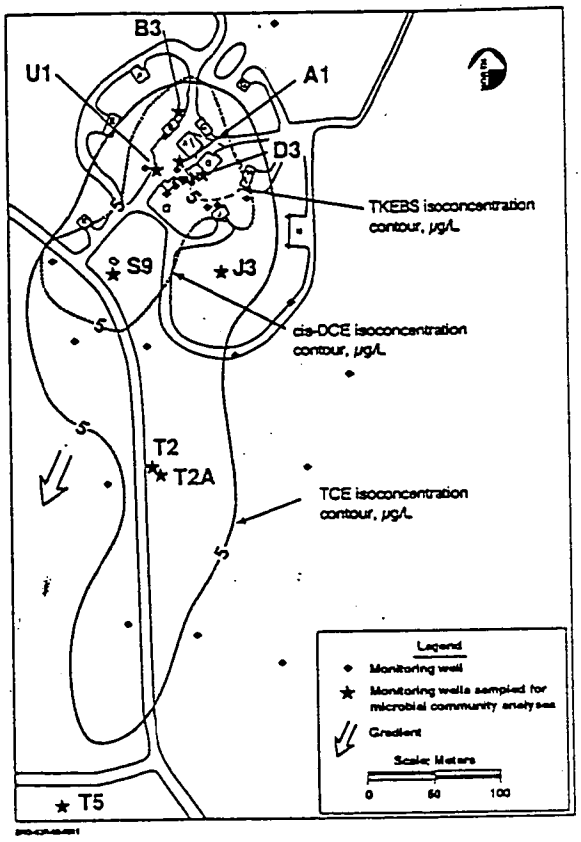


Figure 3. Location of monitoring wells at the Building 834 Operable Unit, LLNL, Site 300, CA

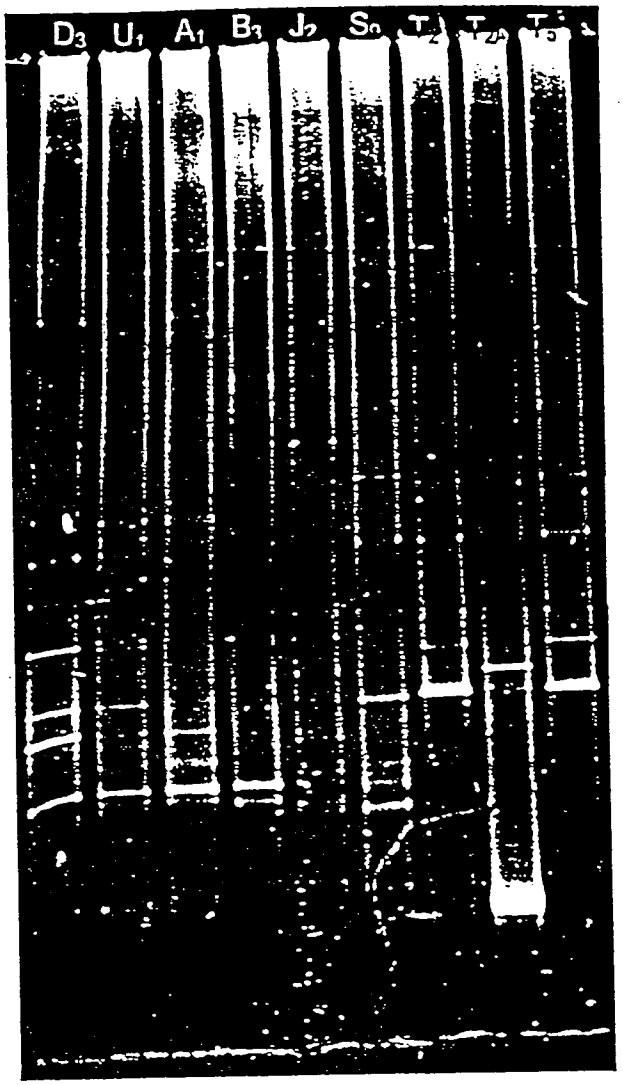


Figure 2. DGGE analysis of 16S rRNA genes amplified from DNA extracted from the wells: D3, U1, A1, B3, J2, S9, T2, T2a, and T5 (Figure 3) using eubacterial primers GC-GM5f and 907r.

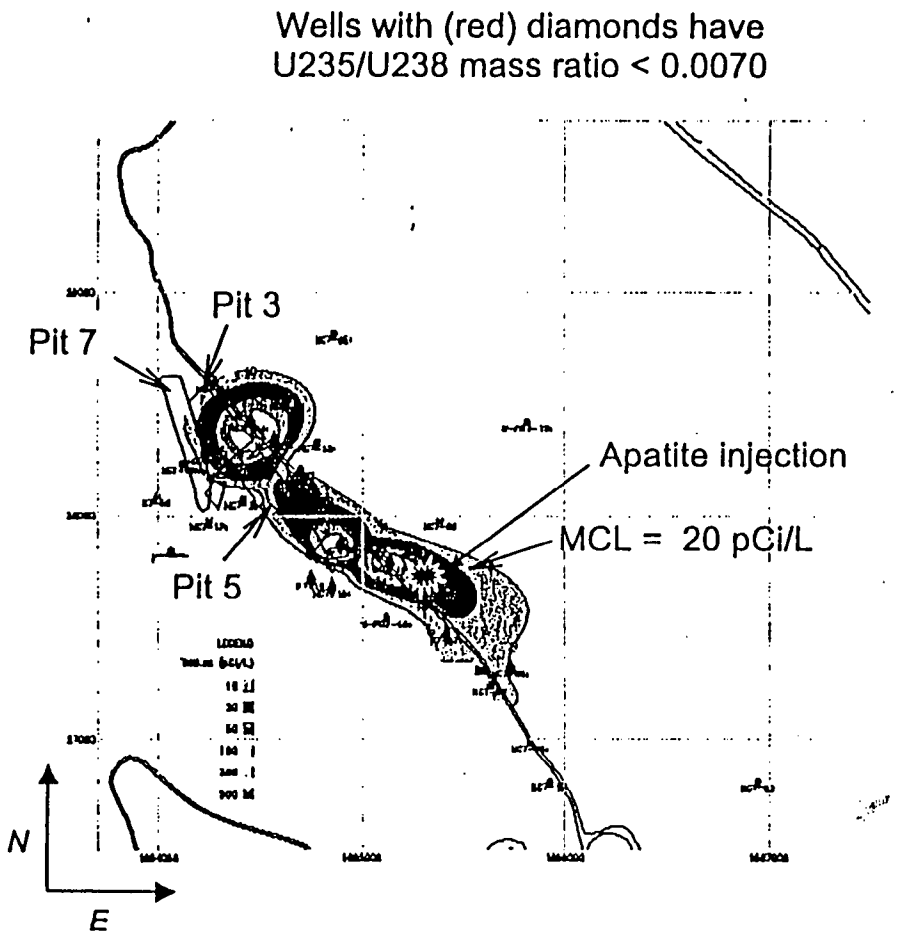


Figure 4. Overview of the uranium contamination, monitoring network and remediation efforts at the Pit 7 Complex, LLNL, Site 300.

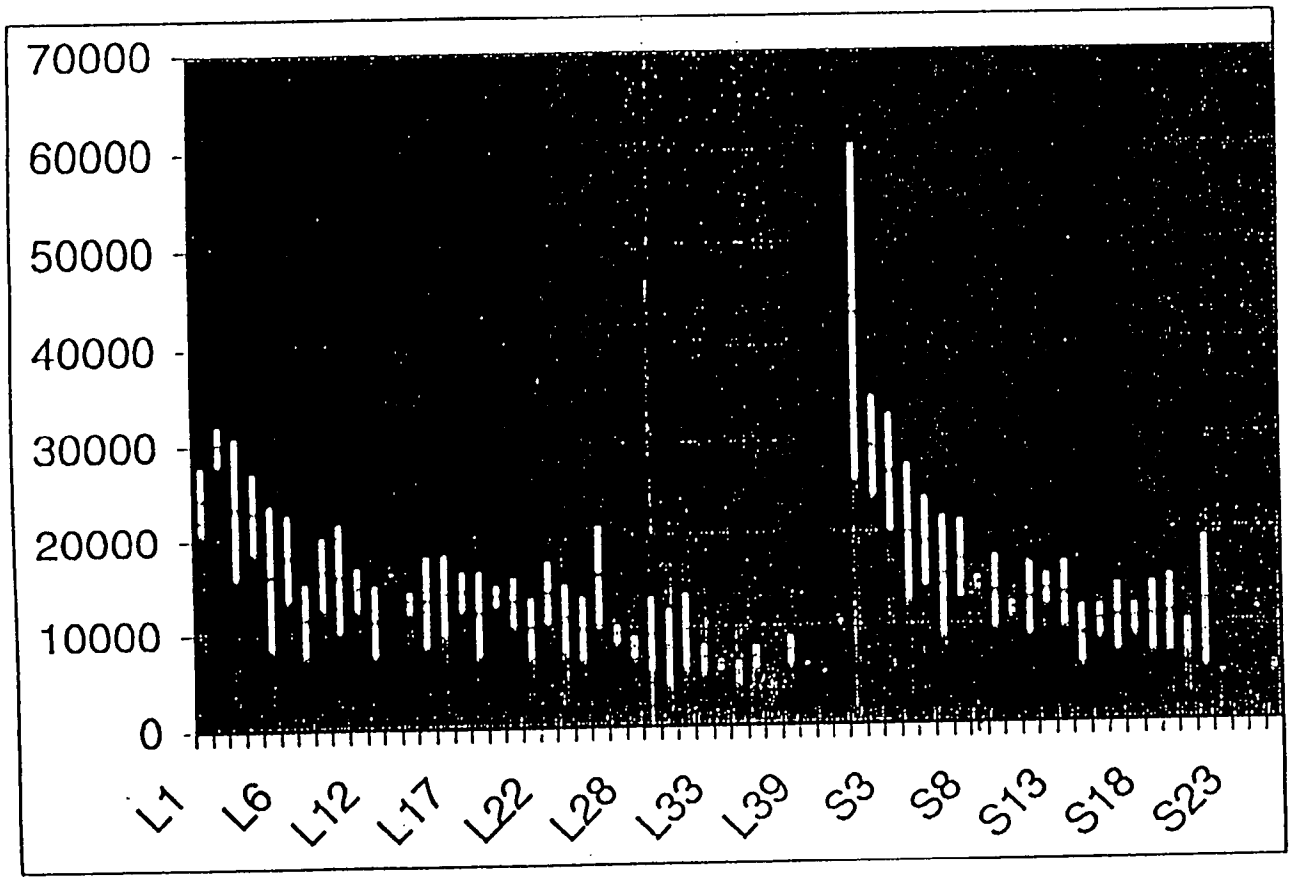


Figure 5. Mass distribution of ribosomal proteins (for all bacterial species in Swiss-Prot ca. 1999) vs ribosomal protein name. Large sub-unit proteins are labeled with an L, small sub-unit proteins are labeled with an S (Not all names are listed due to space constraints). Each horizontal bar represents the mass distribution across different microorganisms. This variability in molecular mass indicates molecule diversity, which is can be exploited in mass spectrometric analyses. Y-axis in units of Daltons. (Source: F. J. Pineda)

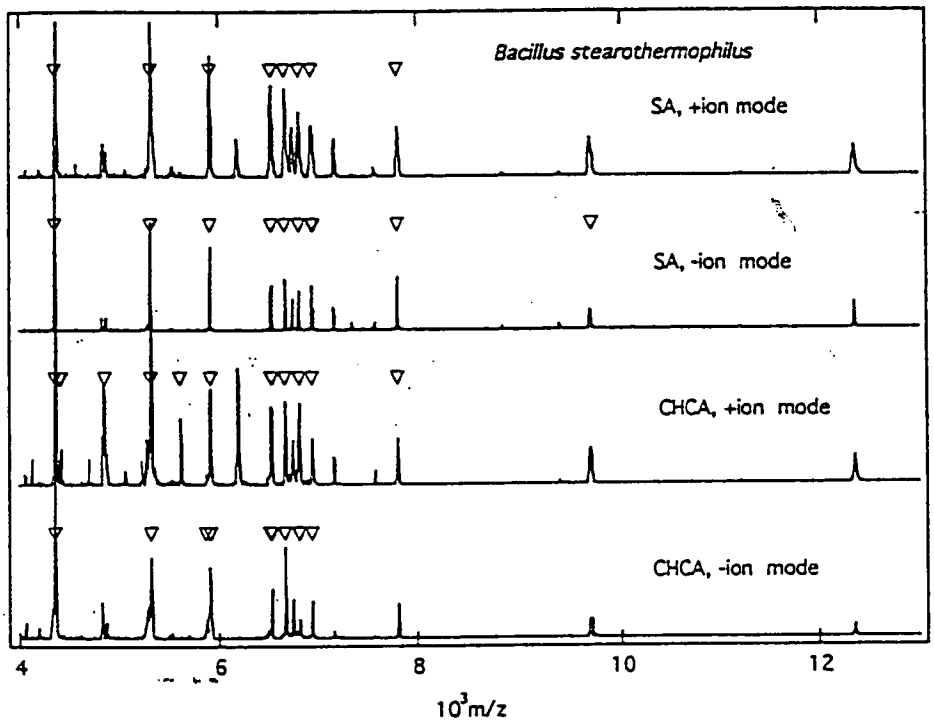


Figure 6. MALDI TOF mass spectra from *B. stearothermophilus* obtained with four different experimental protocols. Peaks that match ribosomal biomarkers are marked with triangles. (Y-axis represents relative signal intensity).

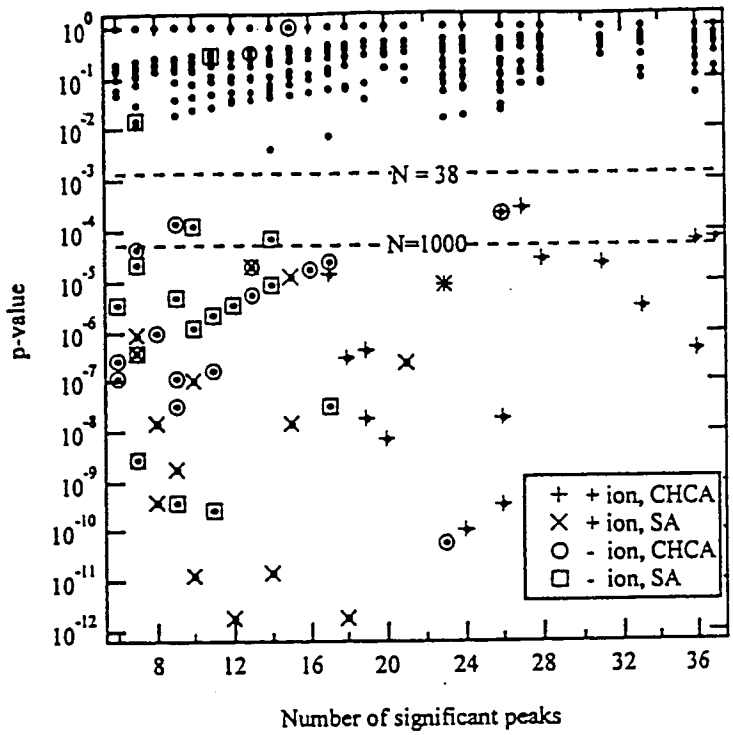


Figure 7. Distribution of the *p*-values for the 3800 database-target microorganism spectra comparisons. Each point represents the *p*-value corresponding to a single comparison. Points corresponding to correct identifications are further labeled by the experimental protocol. Bonferroni-corrected threshold *p*-values for the 95% confidence level for databases with N=38 and N=1,000 microorganisms are marked with horizontal lines. Figure taken from [19].

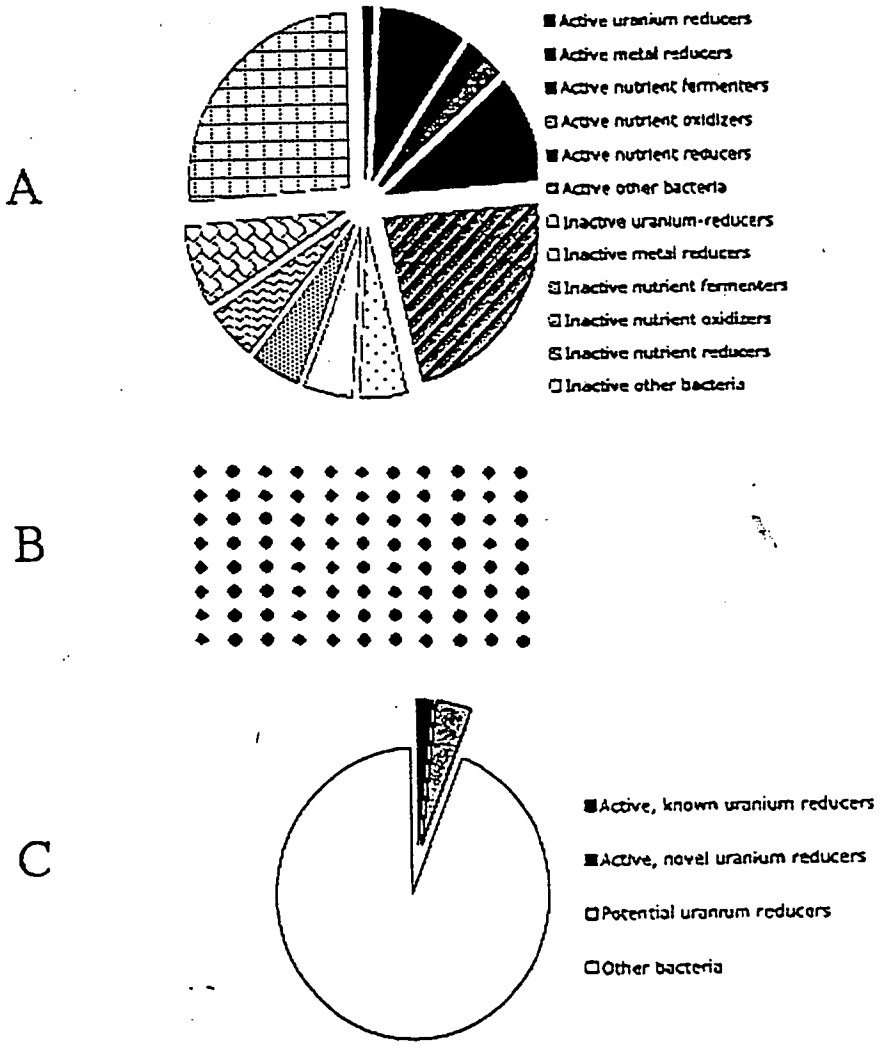


Figure 8.

The above schematic illustrates the utility of the proposed ISBA sampling strategy. Conventional microbial community analysis produces a picture as shown in A; the technique detects the presence of bacteria, however, it does not provide information on their metabolic activity. The use of isotope-labeled nutrients can reveal which of the detected microorganisms are metabolically active (right half of the community shown in A). Use of the proposed ISBA prototype will allow for the determination of up to 96 community profiles determined under various environmental conditions (B). Computational analysis of the resulting data using subtractive community profiling may allow one to identify important pollutant-transforming microorganisms within the large group of active microorganisms (not all metabolically active bacteria are partaking in the bioremediation process). In addition, environmental conditions in the device may allow for the selective enrichment of pollutant-degrading bacteria; some of these may be detected for the first time (C); under appropriate conditions, poorly represented population may be enriched to a level allowing for MALDI TOF MS-based detection/identification. In addition to the microbial profiling data, optional chemical analysis of the proposed device can provide data on the rate and extent of biotransformation in 96 different scenarios, including the conditions prevailing at the site. This information is critical for designing bioremediation strategies for site cleanup.

An important need in bioremediation research is the development of innovative diagnostic technologies that can unambiguously link an observed degradative activity to a specific microbial subpopulation present in the contaminated environment of interest. This invention addresses this need by concentrating on the development and testing of a new solid phase sampling tool, *in situ* microcosm array (ISMA), designed to determine in an automated, standardized, high-throughput mode the composition, dynamics and functions of complex microbial communities and subpopulations, as well as to conduct *in situ* screening studies on nutrient regimes and bioaugmentation strategies suitable for enhancing bioremediative processes; all this being done without altering in any way the chemistry, physics or biology of the contaminated environment under study. Although the proposed tool will have broad applicability to any type of bioremediation, this study will focus primarily on the biotransformation of volatile organic compounds (VOCs) and tetraalkoxysilanes as model pollutants. The device will be tested and evaluated in laboratory and field studies. Specific aims of the proposal are: (a) to produce two identical prototypes of the proposed *in situ* microcosm array sampler and demonstrate their mechanical functionality in laboratory experiments; (b) to evaluate in controlled laboratory conditions whether the ISMA technology can serve to actively capture, concentrate and selectively enrich target microorganisms relevant to bioremediation; (c) to determine whether analysis of ISMA samplers by stable isotope-probing techniques (^{13}C-DNA analysis) allows one to distinguish metabolically active microorganisms—that are directly or indirectly partaking in the transformation of contaminants—from those that are dead, dormant or irrelevant to bioremediation; (d) to evaluate whether ISMA samplers are amenable to analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a rapid analysis technique that potentially can inform about both the identity of microorganisms (genotype) and the molecular composition of cells (phenotype); and (e) to evaluate the applicability of ISMA technology to real-world situations by deploying prototypes of the device at the highly contaminated and well-characterized VOC-containing Superfund site.

B. Selected Peer-reviewed Publications

1. Halden, R. U., and D. F. Dwyer: Biodegradation of Dioxin-Related Compounds: A Review. Bioremediation Journal, 1(1): 11-25 (1997).

2. Vancheeswaran, S., R. U. Halden, K. J. Williamson, J. D. Ingle Jr., and L. Semprini: Abiotic and Biological Transformation of Tetraalkoxysilanes and TCE, c-DCE Cometabolism Driven by Tetrabutoxysilane-Degrading Microorganisms. Environ. Sci. Technol., 33(7):1077-1085 (1999).

3. Halden, R. U., B. G. Halden, and D. F. Dwyer: Removal of Dibenzofuran, Dibenzo-p-Dioxin, and 2-Chlorodibenzo-p-Dioxin from Soils Inoculated with Sphingomonas sp. Strain RW1. Appl. Environ. Microbiol., 65(5):2246-2249 (1999).

4. Halden, R. U., S. M. Tepp, B. G. Halden, and D. F. Dwyer: Degradation of 3-Phenoxybenzoic Acid in Soil by Pseudomonas pseudoalcaligenes Strain POB310(pPOB) and Two Modified Pseudomonas Strains. Appl. Environ. Microbiol., 65(8):3354-3359 (1999).

5. Halden, R. U., B. G. Halden, and D. F. Dwyer: Transformation of Mono- and Dichlorinated Phenoxybenzoates by Phenoxybenzoate-dioxygenase in Pseudomonas pseudoalcaligenes Strain POB310 and a Modified, Diarylether-Mineralizing Bacterium. Biotechnol. Bioeng., 69(1):107-112 (2000).

6. Koester, C. J., H. R. Beller, and R. U. Halden: Analysis of Perchlorate in Groundwater by Electrospray Ionization Mass Spectrometry/Mass Spectrometry. Environ. Sci. Technol., 34(9):1862-1864 (2000).

7. Halden, R. U., A. M. Happel, and S. R. Schoen: Evaluation of Standard Methods for the Analysis of Methyl tert-Butyl Ether and Related Oxygenates in Gasoline-Contaminated Groundwater. Environ. Sci. Technol., 35(7):1469-1474; 1560 (2001).

8. Kane, S. R. , H. R. Beller, T. C. Legler, C. J. Koester, R. U. Halden, and A. M. Happel: Aerobic Metabolism of Methyl *tert*-Butyl Ether by Aquifer Bacteria. Appl. Environ. Microbiol. 67(12):5824-5829 (2001).

9. Lowe, M., E. L. Madsen, K. Schindler, C. Smith, S. Emrich, F. T. Robb, and R. U. Halden: Geochemistry and Microbial Diversity of a Trichloroethene-Contaminated Superfund Site Undergoing In Situ Reductive Dechlorination. FEMS Microbiol. Ecol. 40(2):123-134 (2002).

10. Vancheeswaran, S., S. Yu, P. Daley, R. U. Halden, K. J. Williamson, J. D. Ingle Jr., and L. Semprini: Intrinsic remediation of trichloroethene driven be tetraalkoxysilanes as co-contaminants: results from microcosm and field studies. Remediation 13-14(1):7-25 (2003).

11. Franklin, M. P., Madrid, V., Gregory, S., and R. U. Halden: Spatial Analysis of a Microbial Community Mediating Intrinsic Reductive Dechlorination of TCE to *cis*-DCE at a DOE Superfund Site. To be presented at the 103rd General Meeting of the American Society for Microbiology, Washington, D.C. (2003).

12. Xie, G., T. Palmateer Oxenberg, W. Dong, A. Kalmykov, M. P. Franklin, E. J. Bouwer, and R. U. Halden: Sorption, Bioavailability, and Bioreduction of U(VI) in Sediment from the Aberdeen Proving Ground, MD. To be presented at the 103rd General Meeting of the American Society for Microbiology, Washington, DC, (2003).

13. Halden, R. U., R. N. Cole, C. Bradford, and K.J. Schwab: Rapid Detection of Norwalk Virus-like Particles by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry. 2nd NIH Proteomics Symposium, Bethesda, MD (2003).

An important need in bioremediation research is the development of innovative diagnostic tools that can unambiguously link an observed degradative activity to a specific microbial subpopulation present in the contaminated environment of interest.

Benefits of the tool include the ability to determine in an automated, standardized, high-throughput mode the composition, dynamics and functions of complex microbial communities and subpopulations, as well as to conduct *in situ* screening studies on nutrient regimes and bioaugmentation strategies suitable for enhancing bioremediative processes; all this potentially being done without altering in any way the chemistry, physics or biology of the contaminated environment under study. Although the proposed tool will have broad applicability to bioremediation, this / will focus primarily on the biotransformation of volatile organic compounds (VOCs) and tetraalkoxysilanes as model pollutants. The field site selected for technology testing is the Department of Energy (DOE) Lawrence Livermore National Laboratory (LLNL) Site 300, a VOC- and tetrabutoxysilane-contaminated Superfund site located in Northern California.

The specific aims of this proposal are:

1. To produce two identical prototypes of the proposed *in situ* microcosm array (ISMA) sampler and demonstrate their mechanical functionality in laboratory experiments.
2. To evaluate in controlled laboratory conditions whether the ISMA technology can serve to actively capture, concentrate and selectively enrich target microorganisms relevant to bioremediation.
3. To determine whether analysis of ISMA samplers by stable isotope-probing techniques (^{13}C-DNA analysis) allows one to distinguish metabolically active microorganisms—that are directly or indirectly partaking in the transformation of contaminants—from those that are dead, dormant or irrelevant to bioremediation.
4. To evaluate in a study whether ISMA samplers are amenable to analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a rapid analysis technique that potentially can inform about both the identity of microorganisms (genotype) and the molecular composition of cells (phenotype).
5. To evaluate the applicability of ISMA technology to real-world situations by deploying prototypes of the device at the highly contaminated and well-characterized VOC-containing Superfund site.

Bioremediation. Bioremediation is a promising and rapidly maturing technology potentially allowing for the swift, safe and cost-effective restoration of polluted natural environments (39). When considering bioremediation as a treatment option, one has to examine whether physical, chemical and/or biological constraints exist. Once these have been identified, engineering strategies can be implemented to remove or minimize these limitations. Treatment options include physical, chemical and biological interventions, *e.g.*, hydraulic fracturing of subsurface strata for enhancing water permeability, addition of nutrients, chemical modulators and/or carbon and energy sources for increasing or limiting specific chemical or biological functions, and addition of foreign microorganisms for initiating and/or accelerating pollutant transformation. It is now becoming recognized that at many field sites, a lack of sufficient microbial diversity and/or metabolic potential can explain why biodegradable pollutants fail to undergo biotransformation *in situ* (11). While new findings on the metabolic routes and bottlenecks of degradation are still accumulating (54), it is already clear that the capacity of indigenous microbial populations to adapt to the presence of toxic pollutants and to biodegrade these compounds may be the most important factor in determining the fate of subsurface contaminants (for a review, see (40)). Thus, bioremediation of polluted environments requires a proper understanding of the types of organisms present, what their potential metabolic capabilities are, and to what extent these degradative functions are being expressed *in situ*.

Designing and Monitoring Bioremediation. Contaminated sites across the country may resemble one another but no two sites can truly be identical due to the infinite number of variables describing their characteristics on a micro and macro scale. For this reason, the design of bioremediation strategies requires a

site-specific approach that is typically arrived at by conducting microcosm experiments and small-scale field tests (53). Since both approaches have distinct advantages and limitations, they are often applied in unison (6, 25, 26, 28, 53). Microcosm studies allow one to estimate the rate and extent of biotransformation under simulated site conditions (intrinsic bioremediation) and under enhanced environmental conditions designed to speed up the *in situ* biotransformation process (accelerated bioremediation) (27, 53). Furthermore, they allow for complete mass balance calculations on all constituents contained in the vessel (26). They also facilitate mechanistic studies (53) and provide kinetic data informing about the pace of bioremediation (rate constants) and the treatment endpoint, *i.e.*, the time at which pollutant transformation is complete or ceases to occur (28, 46). Experiments conducted with batch bottles are popular, straightforward, and inexpensive but typically yield estimates of degradation rates that are overly optimistic and not applicable to subsurface environments. Sophisticated flow-through microcosm experiments (column studies) yield more reliable information, however, these come at a much greater financial expense (*e.g.*, (28)). Field studies are even more labor and cost intensive, yet produce the most accurate and reliable information (30). Since field studies are conducted in an open system, mass balance calculations are limited by the quality of the monitoring network. Push-pull experiments (45) are a field study variation requiring only a single monitoring well; they have to be limited in time because of tracer recovery rates that decrease with both time lapsed and groundwater velocity. Due to the limited time an injected test volume of groundwater remains in the subsurface, push-pull tests are not well suited for studying processes requiring long-term adaptation of microbial communities (*e.g.*, nutrient injection-induced anaerobiosis in aerobic saturated subsurface environments). The ideal diagnostic tool for designing and monitoring bioremediation would combine the benefits of both laboratory and field tests. Thus, it would enable one to determine/estimate with reasonable certainty what the causative agents and mechanisms of contaminant removal are, how fast and to what extent pollutants are being removed intrinsically, and what chemicals and/or bacteria need to be delivered to the subsurface to initiate or accelerate biological site cleanup (without itself influencing the environment). In other words, information is required on the microbial community composition, dynamics, functions and requirements at the site.

Microbial community analysis. Today, non-culture dependent molecular-genetic techniques are the standard approach to studying microbial diversity and function. The central dogma of microbial ecology analysis is that only a very small proportion of the bacteria that can be visualized by direct count procedures can actually be cultivated (8, 16, 55). Conventional cultivation techniques incorporate biases, potentially leading to the false conclusion that the few cultivatable organisms recovered from an environment and grown in the laboratory are the most prevalent or most active *in situ.* Advances in molecular biology techniques and microbial phylogeny enable one to identify many organisms in environmental media without the need for cultivation (24), utilizing methods such as analysis of community DNA or RNA directly extracted from environmental samples or by *in situ* microscopy. The use of the 16S rRNA gene as a molecular marker is an established method for determining phylogenetic relationships and for studying ecosystems mainly based on cloning of 16S rDNA fragments amplified from directly extracted nucleic acids (*e.g.*, (34)). While the cloning and sequencing strategies can be labor and cost intensive the recent development of fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) or terminal restriction fragment length polymorphism (t-RFLP) analysis of PCR-amplified 16S rDNA have allowed researchers to look at large numbers of samples in a top-to-bottom approach. *In situ* analysis of microbes has been facilitated by combining advanced microscopic techniques with a range of molecular approaches such as fluorescently labeled probes (FISH, dot-blot hybridization of rRNA), reporter genes or *in situ* PCR (38). The main objective is to gain information on the population structure, compartmentalization of bacterial communities and specific gene expression without disturbing the complex interactions between the cells, *e.g.*, within a biofilm (24). Methods available to analyze the functional diversity and potential of a complex microbial community are less developed than tools to analyze the structural diversity. The limitations of these nucleic acid methods relate to the extraction of the nucleic acids from the environmental samples, biases, and artifacts associated with enzymatic amplification of the nucleic acids, cloning of PCR products, and sensitivity and target accessibility. The easily-produced substrate utilization profiles using BIOLOG microtiter plates allow for intensive spatial and temporal analysis of microbial communities. However, due to the use of complex media and elevated incubation temperatures, the approach is biased towards fast-growing bacteria and thus the metabolic fingerprints are unlikely to resemble the *in situ* metabolic potential (2). Every individual technique has its limitations, in particular when they are applied to complex microbial communities. However, a better

understanding may be obtained by the combined use of molecular methods, microbiological techniques and through characterization of metabolic activities within the given habitat.

The challenge of linking genetic sequences to metabolic activity in situ. Unfortunately, commonly used non-culture dependent techniques such as 16S rRNA gene amplification followed by DGGE and sequence analysis provide only a fraction of the microbial ecology information needed. A critical limitation of this and other approaches is the difficulty of linking a detected strain or species to an observed or suspected metabolic activity. In other words, the ecological role of a detected microorganism remains uncertain. For example, we may know about the presence of a specific microorganism in the subsurface but are left to speculate how it makes a living and how it interacts with other community members. Methods targeting mRNA are more informative in this respect but are too cumbersome for application beyond the high-end research laboratory. Another challenge is that the detection of 16S rDNA sequences in environmental samples does not necessarily imply any of the following: (a) the population of the corresponding microorganism is present, (b) it is physically intact, (c) it is viable, (d) it is metabolically active and (e) it is performing the desired function. Finally, groundwater is the preferred sample matrix for profiling of microbial communities (34), as it is both readily available and inexpensive. Unfortunately, the lifestyle of a given target organism has a significant impact on our ability to detect it in this matrix. In the extreme, a target organism pursuing a sessile lifestyle throughout its existence will be impossible to detect in groundwater at a site even if it is a predominant member of the microbial community. Thus, groundwater monitoring alone may not accurately reflect the microbial community composition and dynamics of subsurface environments. Recently, solid-phase samplers were rediscovered as useful tools for overcoming some of these limitations (17).

Use of solid-phase samplers for microbial sampling. In their simplest configuration, solid-phase samplers are nothing more than a physical surface incubated in an environment of interest for a period of time sufficiently long to allow for the colonization by microorganisms. Buried or submerged glass slides have been used extensively to collect microorganisms from soils, bioreactors and other environments (e.g., (31)). Dr. White from the University of Tennessee, reported on the use glass wool as a passive sampling device (17); the material was lowered into groundwater monitoring wells where it passively collected microorganisms over time. Following retrieval of the tool, microorganisms were extracted from the sampling device and identified via the detection of biomarkers including DNA, phospholipids, fatty acids and respiratory quinones (17). An argument can be made that microorganisms collected with a solid-phase sampler are more representative of the metabolically active microbial community than those obtained by groundwater sampling because the sampling device requires the active physical attachment by the microorganisms to be captured. However, dead microorganisms, cell debris and DNA also may become entrapped. Highly sensitive tools relying on PCR will detect biomarkers of non-living material as well as those of metabolically active microbial community members. Very recently, a novel approach was introduced that exploits stable-isotope markers to distinguish metabolically active microorganisms from those dormant or deceased. This promising technique (41) has not yet been used with solid-phase samplers.

Stable isotope probing and proteomic analyses. Stable isotope labeling techniques and proteomic analyses using mass spectrometry are emerging technologies that offer a way of determining both the identity and metabolic activity of microorganisms. Stable isotope probing (SIP) exploits the fact that the DNA of an organism growing on carbon-13 enriched carbon sources becomes ^{13}C-labeled ("heavier"), thereby enabling one to resolve its DNA from the total community DNA by density gradient centrifugation. The approach was used successfully for the study of methanol-utilizing bacteria in soil (37, 41, 42). The soil of interest was incubated with ^{13}C-labeled methanol, the genomic DNA was extracted and spun down in a gradient of cesium chloride to separate the "heavy" (^{13}C-labeled) DNA from "light" DNA containing primarily ^{12}C. (A small fraction of ^{13}C also is present in "light DNA" as a result of the natural distribution of this isotope in the biosphere.) As stated earlier, this approach is suitable for separating DNA of metabolically active microorganims from that of dormant or dead ones. In addition, the time-resolved analysis of labeling studies allows one to deduce the metabolic activity of a microbial community during a defined period of time. This information in turn can be used to calculate rates of microbial growth and contaminant turnover. Test compounds containing uniformly labeled stable isotopes of carbon, nitrogen, hydrogen, etc. are, however, expensive. Thus, for economic reasons their use will remain restricted to the small scale, such as the miniaturized *in situ* microcosm array

(ISMA) described in this proposal. While representing a powerful research tool, stable isotope probing has limited potential for routine monitoring at contaminated sites: the technique is too time- and labor-intensive. In addition, it may be impossible to automate. An alternative approach for the identification of microorganisms is to look for gene expression products (*i.e.*, proteins) rather than for their characteristic DNA sequences. This can be done with the latest generation of mass-spectrometry instrumentation that offers sufficient speed and sensitivity, while also allowing for complete automation of the analysis process.

MALDI-TOF MS. Matrix assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), with its ability to induce desorption of protein biomarkers from intact bacteria (9), fungi, spores and viruses (56), is a powerful and rapidly emerging technology for fast, portable and robust microorganism identification (13). Initially developed for biodefense applications, this technology clearly also has great potential for public health research (20) and environmental monitoring purposes. MALDI-TOF MS techniques are very rapid (<5 minutes analysis time per sample), have low sample volume requirements (< 1 mL) and have a generic capability to identify microorganisms. The literature ((13) and references therein) indicates that between 5,000 to 10,000 cells need to be present on the sample holder to achieve successful detection. Two recent reviews elaborate on the strengths of this technology and provide an outlook on future applications (13, 32). Robotic devices have been integrated with MALDI-TOF instruments to an astonishing degree. The latest generation of commercially available robotics allows for the fully-automated sample preparation and analysis, including preparation and imaging of 2D gels, harvesting and digestion of the protein spots, and application of the digests to multi-sample MALDI-TOF targets for analysis (33).

This invention integrates the above technologies by providing an innovative strategy for determining microbial community structure, dynamics and functions on the molecular level. In the following, a selected set of preliminary data is being presented to summarize the progress made so far, and to show that our multi-disciplinary research team is well positioned and qualified to carry out the proposed work.

The bioremediation of contaminated sites requires the development of automated, field-ready technologies for studying the complex microbial communities indigenous to contaminated subsurface environments. Moreover, these technologies need to provide evidence at the molecular level suitable for linking metabolic activities observed at the site to the corresponding microorganisms. In order to address this need, we propose the use of an innovative miniaturized down-well device to be analyzed by molecular-genetic and proteomic methods. The proposed ISMA is based on a standard 96-well microtiter format in order to allow for fully automated analysis using commercially available robotics. Each ISMA sampler will hold 96 "capillary microcosms" which can be operated in either batch mode, flow-through mode or a combination of the two.

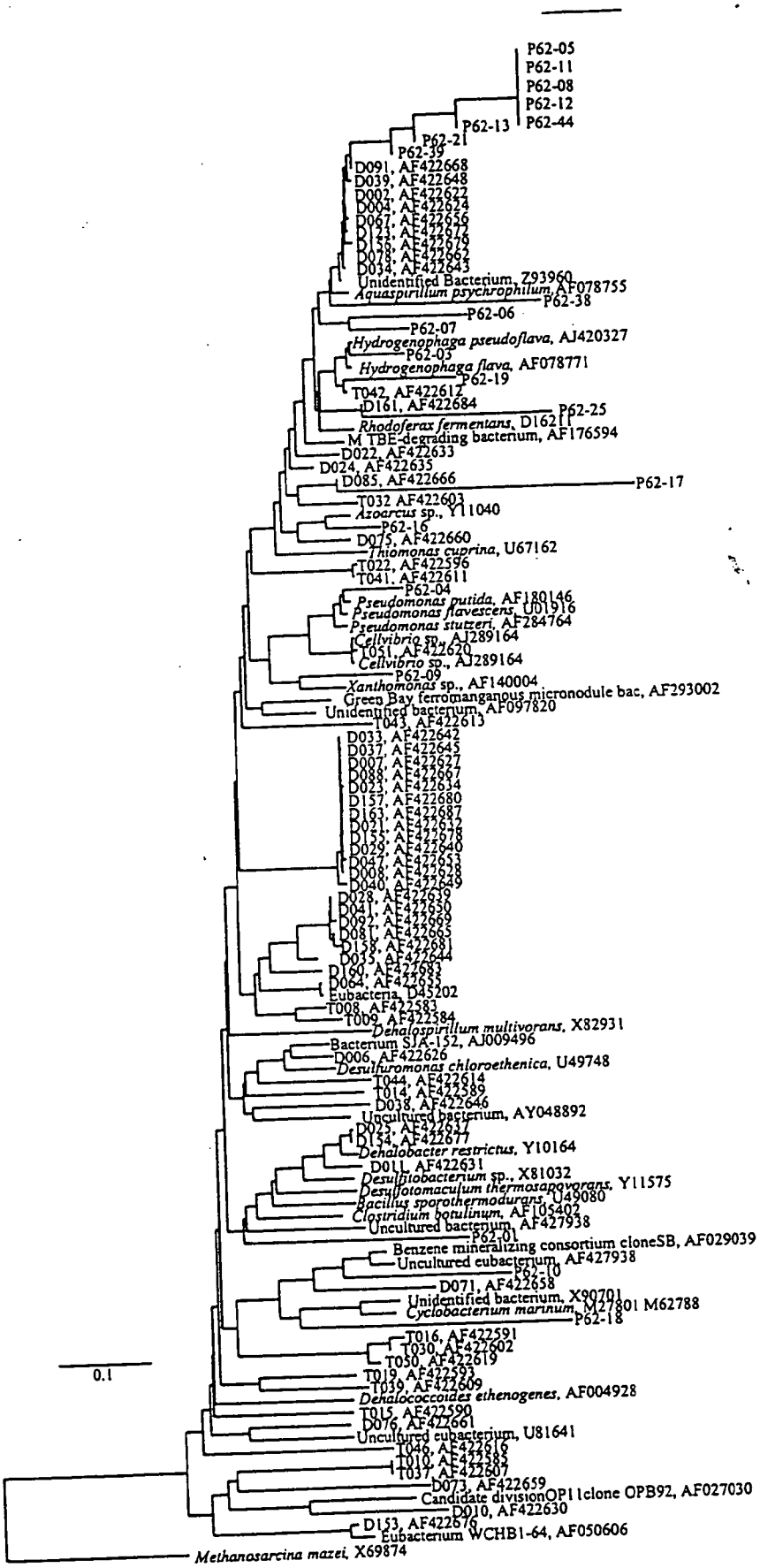


Figure 1. Dendrogram showing published 16S rRNA gene sequences with those obtained by us from groundwater monitoring wells within the Building 834 Operable Unit (Taken from Franklin et al. 2003).

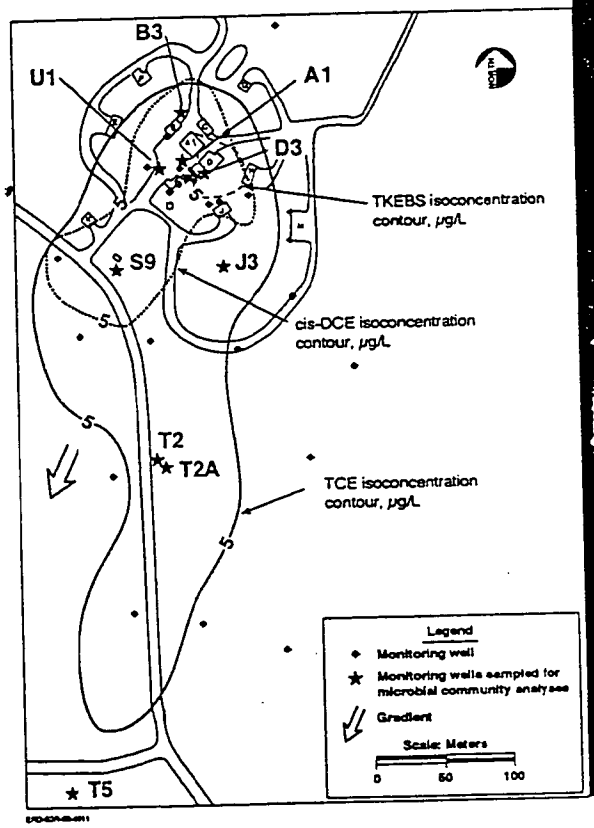


Figure 2.
Field site map indicating the locations of monitoring wells at the Building 834 Operable Unit at LLNL Site 300, CA. Five-ppb-contours show the extent of groundwater contamination caused by spillage of trichloroethene (TCE) and tetrakis(2-ethylbutoxy)silane (TKEBS). The contour for *cis*-1,2-dichloroethene (*cis*-1,2-DCE) indicates the presence of intrinsic reductive dechlorination activity at the site.

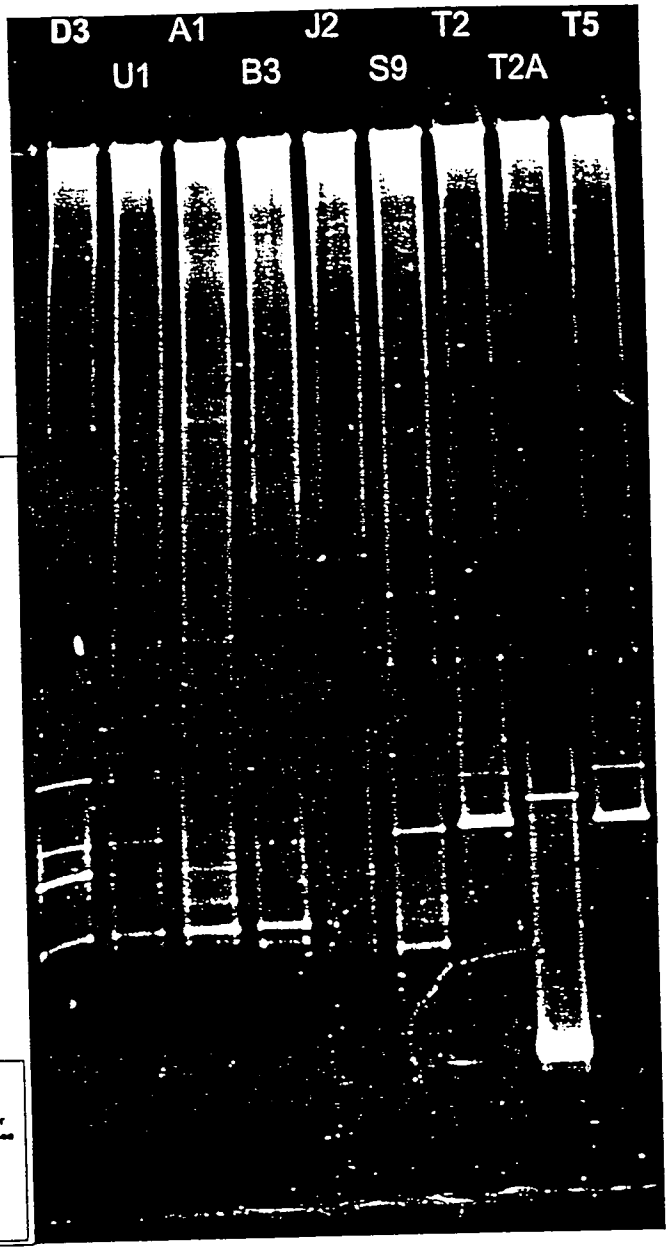


Figure 3.
Composite digital image of an ethidium bromide-stained DGGE gel (35-55% denaturant) separating the bacterial DNA fragments coding for 16S rRNA. Using the primers GC-GM5f and 907r, fragments were amplified directly from DNA extracted from the following groundwater monitoring wells at LLNL Site 300: W834-D3, -U1, -A1, -B3, -J2, -S9, -T2, -T2A, and -T5.

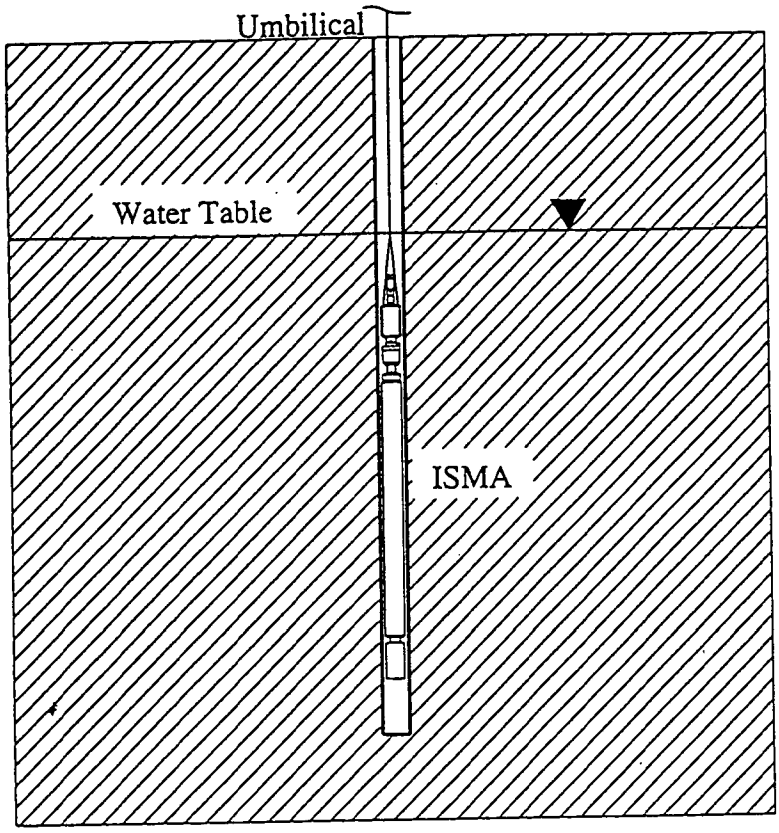
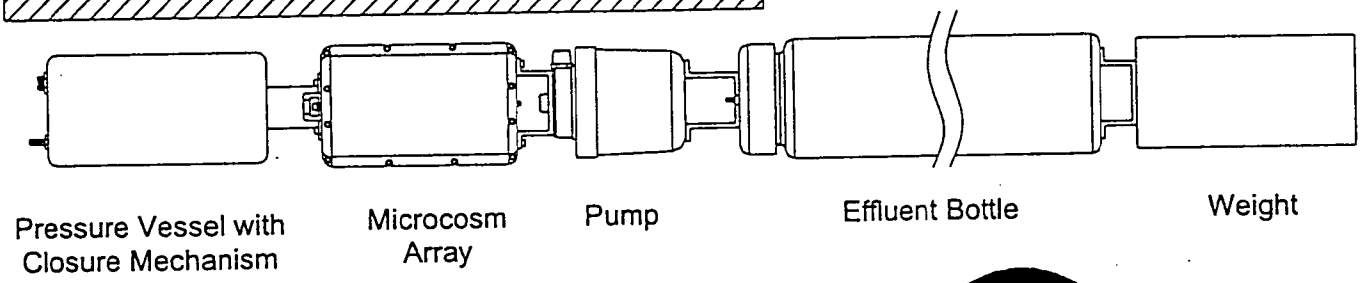


Figure 4.
Schematic showing the *in situ* microcosm array (ISMA) suspended in a standard 100-mm diameter groundwater monitoring well. The device is supported from the surface via an umbilical, that holds it in place and provides power and vacuum for actuation of the integrated closure mechanism and pump.

Figure 5.
In situ microcosm array (ISMA) system components.



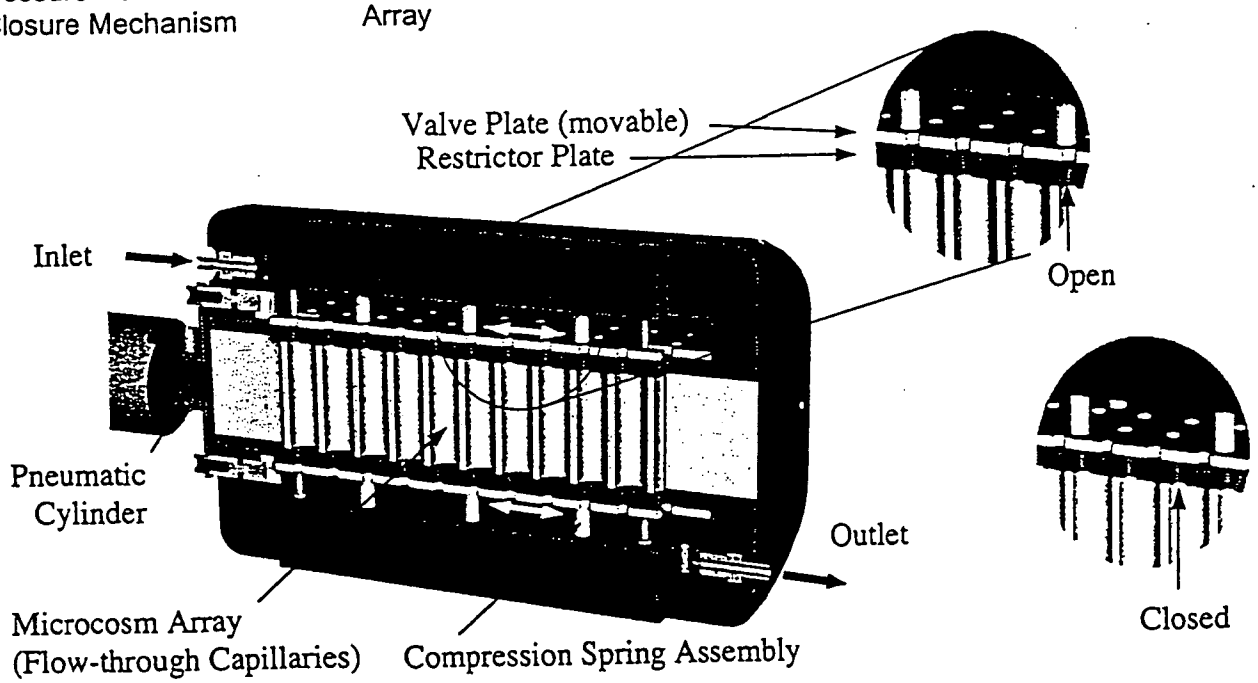


Figure 6. Cutaway view of the closure mechanism and the microcosm array (see Section D for details).

The device is deployed by lowering it into a monitoring well to the desired depth below the water table. The umbilical tether connecting the ISMA to the surface allows one to send an electrical signal to the built-in closure mechanism and the integrated water pump. Triggering of the device from the surface will cause the two valve plates to shift and the pump to start, thereby exposing each of the 96 "capillary microcosms" to a constant flow of groundwater. Microorganisms suspended in the groundwater will be forced into the capillaries. Each capillary is filled with a filtration material (*e.g.*, inert glass wool plugs). The filtration matrix can be amended with test substances diffusing from an inert polymer matrix (*e.g.*, chunks or beads of noble agar containing microbial carbon and energy sources). Some of the capillary microcosms will contain stable isotope labeled substances to facilitate stable isotope probing (SIP) analysis. The device can be operated for days or weeks in flow-through mode. Alternatively, the closure mechanism can be activated after an initial inoculation period in order to continue incubation in batch mode. Switching from flow-through mode to batch mode may be required to simulate anaerobic conditions in saturated aerobic environments. The effluent of the various capillary microcosms is collected in a bladder at the bottom of the device, with a check valve preventing backflow of liquids. Owing to the presence of a collection bladder and the unidirectional flow within the device, none of the effluent can escape into the surrounding groundwater. For this reason, the device allows one to test radiolabeled compounds, stable isotope compounds, toxic chemicals and even foreign microorganisms under *in situ* conditions without releasing any of these agents into the environment.

During *in situ* incubation of the device, all bacteria directly or indirectly involved in the utilization of isotope-labeled electron donors may become enriched in stable isotope-labeled DNA. Following retrieval of the tool from the well, microorganisms will be extracted from the capillary microcosms and, if applicable, their isotope-labeled, higher-density DNA will be separated from background DNA by density-gradient centrifugation. This higher-density DNA (and the non-labeled DNA) will then be analyzed using standard molecular techniques described in detail below. Additional analysis on replicate capillary microcosms containing non-labeled substrates will be analyzed by MALDI-TOF MS. By taking advantage of the 96-well format of the device, this analysis can potentialy be automated using commercial robotics for the extraction and purification of proteins on two-dimensional gels, and the spotting of purified digested extracts on the MALDI target. Obviously, the effluent of each capillary microcosm and the content of the capillary itself could be analyzed chemically to determine biotransformation activity and to calculate the rate and extent of bioremediation under a given set of conditions. However, this work is outside of the scope of this exploratory research project.

Two prototypes of the device will be manufactured by the Instrument Design Group (IDG) of the Johns Hopkins University (19). The device will first be tested mechanically in the laboratory to ensure its proper operation. Thereafter, we will load the compartments of the ISMA with non-labeled or labeled compounds, and conduct proof-of-concept tests in the laboratory using defined mixed cultures as surrogates for more complex environmental microbial communities of unknown composition. These experiments will be designed to test the ability of the device to capture and concentrate suspended microorganisms entering the capillary microcosms in a controlled flow of simulated groundwater. Thereafter, we will add uniformly ^{13}C-labeled compounds ($^{13}C_6$-labeled benzoic acid) to some of the flow-through microcosms by mixing noble agar pieces containing the test compound into the filtration material of the test compartment (noble agar pieces mixed into the glass wool plug). Following inoculation and incubation of the so prepared ISMAs, we will extract the DNA, perform density gradient centrifugation to separate the "heavier" labeled DNA from the "lighter" non-labeled DNA, amplify a 16S rRNA gene fragment using universal eubacterial primers, and analyze the purified products of amplified light (^{12}C-enriched) and heavy (^{13}C-enriched) DNA by DGGE. These experiments are designed to determine whether the use of isotope-labeled compounds in conjunction with stable isotope probing can allow one to distinguish dormant or dead microorganisms from those that have been metabolically active within the device during the incubation period.

Additional experiments will focus on the applicability of MALDI-TOF MS as a tool for rapid and automated analysis of mixed cultures focusing on abundant proteinaceous biomarkers that yield information on both the identity (genotype) of the organism (targeting ribosomal proteins) and the expression of functional

genes (phenotype; targeting enzymes relevant to bioremediation). MALDI-TOF MS experiments will be performed at the proof-of-concept level concentrating on monocultures and defined communities of up to 5 members. Our analysis will focus on putative ribosomal proteins as characteristic biomarkers revealing the genotype of detected organisms. In order to avoid the need for extensive protein cleanup steps, this work will be conducted with intact cells of pure cultures. For the evaluation of functional gene products, we will focus on the detection of a well-characterized enzyme of importance to the bioremediation of nitroaromatics, the nitroreductase of *Enterobacter cloacae* (29). This protein can be overexpressed in *E. coli* yielding the target protein at concentrations (of up 65.7 mg/L; (29)), potentially high enough to allow for direct detection of the protein in intact cells or in crude extracts of *E. coli* by MALDI-TOF MS. If necessary, proteins will be partially purified from crude cell extracts using published protocols (e.g., (29)). The literature and our data on virus detection by MALDI-TOF MS (21) indicate that analysis of monocultures may not require any protein purification but may benefit from the analysis of trypsin digests for identification of targets by characteristic peptide digestion fragments/sequences (*e.g.*, (21, 56).

The final objective of this study is to demonstrate the usefulness of the ISMA technology in field situations. For this purpose, we will deploy our ISMA prototypes at the Building 834 Operable Unit at Site 300, CA. These tests will be conducted in two monitoring wells (W-834-D3 and -T5; Fig. 2) for which we have collected an extensive chemical and ecological dataset (18, 34, 46, 51-53) (see Figure 1). In these experiments, we will use ^{13}C-labeled tetraalkoxysilanes and some of their ^{13}C-labeled biological breakdown products (*e.g.*, 2-ethylbutanol, 2-ethylbutyric acid, butanol, butyric acid and acetate; see (53) for details on the metabolism of these compounds) in order to gain insights into which bacteria are involved in the breakdown of this group of chemicals that act as potent drivers of *in situ* reductive dechlorination at the study site (46, 51-53).

D3-SA#1: Specific Aim #1. To produce two identical prototypes of the proposed in situ microcosm array (ISMA) sampler and demonstrate their mechanical functionality in laboratory experiments.

The JHU Instrument Design Group will manufacture two prototypes of the proposed device according to the drawings shown in Figs. 4-6. All materials used will be inert and solvent resistant in order to allow for surface sterilization of the device prior to its deployment. Proposed materials for the various components (Fig. 5 and 6) are Teflon® (microcosm array), Viton® (restrictor plate), stainless steel coated with Teflon® (pressure vessel, other structural components), Delrin® (a synthetic stone; microcosm array housing) and Kevlar® (umbilical tether). The dry mass of the ISMA system is ~7.2 kg (15 lbf). With the 5L-effluent bottle attached, the device has an overall length of 173 cm (68 inches).The dry mass on a subsystem basis is as follows: array and closure mechanism ~1.7 kg (3.8 lbf); effluent bottle ~1 kg (2.2 lbf); pump ~1.8 kg (4 lbf); weight ~2.7 kg (6 lbf). When the effluent bottle is full the mass increases by 5 kg resulting in a total wet mass of the system of 12.2 kg. We will conduct mechanical tests on the ISMA closure mechanisms and the integrated pumps prior to use of the devices in the laboratory and in the field. Pump tests will include the generation of flow rate curves for the integrated variable speed pumps (1 per device) for calibration purposes. The ISMA is designed to remotely extract and culture environmental samples for bioremediation studies. It is narrow enough to fit into a standard 100 mm (4") diameter monitoring well at depths up to 100 m (~300 ft). Conservative estimates for pressure tolerance indicate that the device can be submerged more than 10 m (>30 ft) below the water table (see Fig. 4 for an illustration). More detailed computer analyses and tests will have to be performed to determine the true depth limit. In any event, these specifications already far exceed the maximum saturated thickness (<20 ft) in the shallow perched water-bearing zone of interest (18).

As shown in Fig. 5, the ISMA system consists of a pump, a 96-well microcosm (microtiter) array, a closure mechanism, an effluent bottle, and a weight. The system functions as follows. The device is filled with sterile water and lowered into the well with the array sealed. The closure mechanism is opened via a pneumatic cylinder and groundwater is quickly transferred to the microcosm array by a pump operating initially in high-flow mode to replace the sterile water with groundwater (~120 mL dead volume). Following replacement of three dead volumes, the flow rate is being reduced to the groundwater velocity at the site (typically ~0.5 ft/day). Groundwater flows through the array exiting into the effluent bottle. Air displaced from

the effluent bottle is allowed to escape via a long piece of tubing rising along the tether to a height above the water table. Microorganisms are trapped in the webbing placed inside the capillary microcosms as the groundwater flows through the device. When the effluent bottle is full, a float trips power to the pump and actuates the pneumatic closure mechanism sealing the array. Immediately, or after an additional incubation period in batch mode, the device is removed from the well and taken to the lab where the array is extracted and samples are analyzed.

The ISMA utilizes a closure mechanism (Fig. 6) to remotely seal the 96 capillary microcosms after a sufficient groundwater volume has been passed through the device. Each microcosm measures 7.5 mm in diameter and 25 mm in depth, yielding a working volume of 1,100 µl. As shown in the Fig. 6, flow is injected into the mechanism via a single inlet thereby filling the upper cavity above the valve plate. In the open configuration (Fig. 6, upper inset), the holes in the upper and lower valve plates are aligned with holes in the underlying restrictor plates allowing fluid to pass through the array. Flow exits from the lower cavity via a single outlet. To seal the array, the valve plates are translated horizontally blocking the holes in the restrictor plate and cutting off the flow (Fig. 6, lower inset). The valve plates are translated using a pneumatic cylinder. A compressive force is applied to the valve plates using springs to facilitate a tight seal between the valve and restrictor plates. The valve plate will be made of stainless steel to provide the necessary rigidity and coated with Teflon® to allow for smooth valve operation. The closure mechanism is actuated using a pneumatic cylinder. Pressure is supplied to the cylinder locally using a small pre-charged pressure vessel. In order to keep the unit compact, the pneumatic cylinder, control valves, and position sensors are encased in the pressure vessel. Prior to deployment into the well, the vessel is charged. A low voltage electrical feed from the surface is used to switch the state of the control valves; thus opening/closing microtiter valve plates. Signals from the position sensors on the cylinder are fed to the surface indicating the state of the device. Bleed-off air from the cylinder is vented above the water table via a tube in the umbilical.

D3-SA#2: Specific Aim #2. To evaluate in controlled laboratory conditions whether the ISMA technology can serve to actively capture, concentrate and selectively enrich target microorganisms relevant to bioremediation.

All 96 positions of each of the two ISMA devices will be loosely stuffed with sterile glass wool plugs using surface sterilized tweezers following which the devices will be assembled for testing. Five different strains of bacteria (*E. coli*, *Bacillus subtilis*, *Deinococcus radiodurans*, *Rhizobium meliloti* and *Pseudomonas* sp. Strain B13-D5) will be grown in the dark (rpm, 30°C) on either Luria-Bertani (LB) medium (36), TYP medium (15), TYC medium (47), or M9 minimal medium (36) containing appropriate carbon and energy sources (5 mM) and trace minerals solution (22). Cells will be harvested by centrifugation (10,000 *g*; 20 min), washed twice with phosphate buffered saline (PBS, (36)), and resuspended in PBS to a final density of approximately 5 x 10^4 CFU/mL. Equal aliquots of the individual bacterial suspensions will be combined to produce a defined 5-membered community containing approximately 1 x 10^4 colony forming units (CFU) of each of the five different bacteria per milliliter. This mixed culture (1.5 L) will be placed in a large Erlenmeyer flask equipped with a magnetic stir bar for mixing (80 rpm; 4°C). Equal amounts (500 mL each) of the suspension will be fed at a low flow rate into the inlets of the two ISMA prototypes using the integrated pumps. At the end of the inoculation period, the device will be drained and opened. Glass wool plugs will be removed aseptically, the capillary washed through with sterile PBS and the trapped microorganisms extracted from the plug by vigorous shaking (1 hour) in test tubes containing 5 mL of PBS (including the above rinse volume). Extracted bacteria will be diluted and enumerated on appropriate selective media using standard microbiological techniques that we have applied in the past (*e.g.*, (22, 23)). The total volumes passed through the two devices will be recorded. Influent samples (periodically taken) and a composite effluent sample will also be examined to determine their individual cell densities. The latter measurements will allow us to determine average flow rates, total number of bacteria fed into the device and the fraction of bacteria captured by the ISMA.

Next, the above experiment will be repeated using ISMAs equipped with a carbon and energy source suitable for selectively enriching *Pseudomonas* sp. Strain B13-D5, one of the five test strains. ISMA samplers will be equipped with glass wool plugs containing a defined amount of small agar cubes measuring about 1

mm in length. Individual compartments will contain approximately 10% agar by volume. The agar cubes will be prepared by cutting agar slabs prepared with M9 minimal medium (36) containing trace elements (22), 2% Noble agar, and either no added carbon sources or benzoic acid (5 mM) as a carbon and energy source for enrichment of Strain B13-D5 (22). These two types of agar will be placed in the ISMA in alternating sequence, so that half of the compartments of the device contain agar with the carbon source. Again, a feed solution (10^3 CFU/ml of each of the five model organisms in 10-fold diluted M9 buffer) will be passed through both devices. In this experiment, the same overall mass of bacteria will be delivered to the ISMAs a in the previous experiment, however, over a 10-fold longer period of time. This additional incubation period may facilitate growth of Strain B3-D5 on the benzoic acid presented in the device. The ISMA will be analyzed as described above in order to determine whether it indeed can provide a micro-environment for the selective *in situ* enrichment of bacteria of importance to bioremediation.

D3-SA#3: Specific Aim #3. To determine whether analysis of ISMA samplers by stable isotope-probing techniques (^{13}C-DNA analysis) allows one to distinguish metabolically active microorganisms—that are directly or indirectly partaking in the transformation of contaminants—from those that are dead, dormant or irrelevant to bioremediation.

Cells will be extracted from the ISMA as described above. Stable isotope-labeled and non-labeled crude DNA will be extracted from the samples using the alkaline lysis method described by Schauer (44). The crude extract will be fortified with non-labeled DNA from *Sphingomonas* sp. Strain RW1 in order to simplify visualization of DNA bands in subsequent steps. The ^{13}C-labeled "heavier" DNA will be resolved from the non-labeled "lighter" DNA according to the method described by Radajewski (41) using cesium chloride ethidium bromide density gradients. The crude DNA extracted will be analyzed by amplifying target genes, such as the 16S ribosomal genes and by separating them according to DNA sequence using DGGE. The 16S rDNA will be enzymatically amplified from the crude DNA according to Teske and co-workers (48) and the resultant amplified DNAs compared using DGGE (43).

This method will give us the microbial community fingerprint for those organisms metabolizing the isotope-labeled substrate. Those organisms utilizing the labeled substrate will be identified by excising the DNA from the gels using sterile pipette tips for sequential phylogenetic analysis. Fractions of acrylamide gel containing the DNA will be incubated in 100 µl of sterile dH20 at 4°C overnight. A 1-µl aliquot of this solution will be used for subsequent PCR amplification. PCR products generated by using the DGGE primers are re-run under the same conditions to confirm the purity of the DNA on another DGGE gel and the PCR product will be purified with the QIAquick-spin DNA purification system (Qiagen) as per manufacturers instructions. The cleaned PCR product will be subjected to cycle sequencing.

DNA Extraction. In short, the bacteria will be collected as above and then concentrated using centrifugation, 2 ml of lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 sucrose and 50 mM Tris HCl pH 9.0) will be used to resuspend the bacteria captured by the glass wool plug, and this suspension will then be incubated at 37°C for 45 min. Proteinase K 0.2 g/ml plus SDS 1% will be added and suspension incubated at 55°C for 60 min. The lysate will be recovered and extracted twice with equal volumes of phenol-chloroform-iso amyl alcohol (25:24:1, pH 8). Excess phenol will be removed by the addition of an equal volume of chloroform. The aqueous phase will be removed carefully and using isopropanol and sodium acetate the DNA will be precipitated, washed with 70% ethanol and resuspended in 300 µl of sterile distilled water (14).

PCR. The 16S rDNA will be amplified from the crude DNA according to Teske (48) and the resultant amplified DNA compared using DGGE (43). These same methods will also be implemented to analyze controls consisting of glass plugs that had not been inoculated. The primer combination GM5f-GC (forward) and 907r (reverse) amplifies an approximately 550 bp fragment of the 16S rRNA. The nucleotide sequence of the forward primer, which is specific for eubacteria (5'-CCTACGGGAGGCAGCAG-3') contains at its 5' end a 40 base GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3') to stabilize the melting behavior of the DNA fragments. The reverse primer used targets the universal consensus sequence (5'-CCCCTCAATTCCTTTGAGTTT-3'). A "touchdown" PCR (10) will be used, in which the annealing temperature is set at 65°C and decreases by 0.5°C every cycle until a touchdown of 55°C, at which

temperature a further 10 cycles are carried out. PCR amplification will be performed in a total volume of 50 µl in a 0.2 ml microfuge tube. Each tube will contain 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 9.0), 2% bovine serum albumin (BSA), 100 pmol each dNTP, 50 pmol of each primer and 1 unit of Redtaq genomic DNA polymerase (Sigma, MI). Template DNA at a concentration of 1 ng will be added to the reaction mix. The PCR machine to be used is a PTC-2000 DNA Engine Peltier Thermal Cycling System, MJ Research, MA. PCR products will be identified on 1% agarose gels stained with ethidium bromide and visualized using a UV transilluminator and a gel documentation system (Alpha Imager 2000 V5.5, Alpha Innotech Corporation,VA). Amplified DNA of the correct size will be reconditioned according to Thompson (49), that is, a low cycle number reamplification of a ten-fold diluted template PCR product will be performed to reduce the potential for formation of heteroduplexes.

DGGE. Fifty µl of reconditioned PCR product will be loaded onto the denaturant gradient gel for analysis of the microbial assemblage of PCR fragments obtained by amplifications of the DNA extracted from the compartment. The DGGE analysis will be performed as described by Schafer and Muyzer, (43), with 6% (wt/vol) acrylamide gels (in 0.5 TAE: 20 mM Tris acetate [pH 7.8], 10 mM sodium acetate, 0.5 mM disodium EDTA) containing a linear chemical gradient ranging from 35% to 60 % denaturant. Gels will be poured from 6 % (wt/vol) acrylamide stock solutions (acrylamide-N,N-methylene-bisacrylamide, 37:1) containing 0 and 100 % denaturant (7 M urea and 40 % [vol/vol] formamide, deionized with AG501-X8 mixed-bed resin [Bio-Rad Laboratories, Inc.]). The gels will be run for 18 hrs at 60°C and 100 V. Bands will be visualized by staining. The optimal method for staining DGGE gels is ethidium bromide staining, the gel is stained in 100 ml of 1 x TAE buffer containing 50 µg/ml ethidium bromide. This is gently agitated for 15 min, the solution discarded and replaced with distilled water to remove excess stain and left for 10 min before pouring off the remaining liquid. The gels will be visualized with a UV transilluminator and a gel documentation system (Alpha Imager 2000 V5.5, Alpha Innotech Corporation, VA).
This technique has been widely used to determine the genetic diversity of natural microbial communities (43), in this case we are using it to analyze the influence of our sampling methods on the obtained picture of the natural community. Some bands will be extracted from the DGGE analysis for sequencing in order to validate the process.

D3-SA#4: Specific Aim #4. To evaluate in a proof-of-concept study whether ISMA samplers are amenable to analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a rapid analysis technique that potentially can inform about both the identity of microorganisms (genotype) and the molecular composition of cells (phenotype).

The strategy for this task is as follows: (a) determination of specific biomarkers for 5 model organisms, (b) determination of minimal cell quantities required for detection of specific organisms by MALDI-TOF MS, (c) analysis of selected samples containing defined mixed cultures of microorganisms, (d) analysis of cells of *Pseudomonas* Strain B13-D5 and *E.coli*, not induced and induced for overexpression of phenoxybenzoate dioxygenase (POB dioxygenase) (7) and nitroreductase (29), resepectively. (a) We will obtain MALDI-TOF mass spectra of intact cells from pure cultures of *E. coli*, *Bacillus subtilis*, *Deinococcus radiodurans*, *Rhizobium meliloti* and *Pseudomonas* sp. Strain B13-D5 grown on appropriate minimal media and on complex media (*e.g.*, Difco nutrient broth) using a Voyager DE-STR MALDI-TOF mass spectrometer and established protocols (9). Prominent mass spectral peaks (typically 10-30 for intact cells) that are detectable in the 4,000 to 15,000 *m/z* range (irrespective of growth conditions) will be tabulated for each organism. Candidate biomarkers that are detected in more than one type of organism will be discarded. This initial peak selection process is designed to maximize the odds of focusing on proteinaceous biomarkers, and more specifically on singly charged ions from intact ribosomal proteins that are known to dominate mass spectra of intact bacterial cells (see (3, 12) and references therein; ribosomal proteins contribute up to 21% to the total protein content of *E. coli*, for example (4)). Dr. Pineda of the Johns Hopkins University is currently developing a Network-enabled Microorganism Identification (NEMID) database tool that predicts ribosomal biomarkers for microorganisms based on sequencing data, accounting for potential *N*-terminal methinine loss (personal communication). If available at the time of this study, we will make use of this tool to extract ribosomal biomarker peaks from our generated list of potential candidate peaks. Our selection of microorganisms will work to our advantage because 4 of the 5 microorganisms proposed for these studies have already been completely sequenced

(Strain B13-D5 represents the one exception). (b) We will experimentally determine the minimal number of cells required to successfully obtain a mass spectrum featuring 3 or more characteristic biomarkers; the literature indicates that a minimum of about 10,000 cells need to be present on the sample holder to achieve successful detection. (c) If the detection limit is sufficiently low, we will analyze the samples generated in experiments described in Specific Aim #2. Specifically, within a background of other microorganisms, we will attempt to detect by MALDI-TOF MS cells of Strain B13-D5 that have been selectively enriched in the ISMA during growth on benzoate-containing agar. (d) We will analyze by MALDI-TOF MS cells of Strain B13-D5 not induced and induced for POB dioxygenase (22), consisting of 46.3 and 33.6 kDa subunits. Similarly, we will attempt to detect the *Entereobacter cloacae* nitroreductase by comparing mass spectra from induced and not induced cells of *E. coli* pRK1 focusing on the 29 kDa nitrogenase (29). It is likely that the functional enzymes cannot be detected directly because of their lesser abundance and unfavorably large mass. Therefore, we are prepared to selectively extract and partially purify proteins from cell extract using the standard techniques outlined below. Purified proteins will be digested with trypsin and obtained data analyzed using SwissProt and NCBI database searches using our established methods for microorganism detection (20).

Proteins will be extracted from microbial cells with 35% acetonitrile/1% trifluoroacetic acid (TFA) and bound to a strong cation exchanger macrospin column (SMM-SCX, The Nest Group, Inc.). Proteins will be eluted from the macrospin column in at least four ammonium chloride salt steps ranging from 10 mM to 1 M ammonium acetate or ammonium chloride. Fractions will be dried, resuspended in 150 mM sinapinic acid (SA) containing 35% acetonitrile/1% TFA and analyzed by MALDI-TOF MS (Voyager DE-STR, Applied Biosystems, Inc) if necessary, in both negative and positive ion mode to enhance the discriminatory power of the assay. When necessary, salt fractions will be desalted using C4 Ziptips (Millipore Corp., Milford).

Reverse-phase chromatography represents an alternative to ion exchange chromatography and will be tested using C8 Macrospin columns (SMM-SS08V, The Nest Group, Inc.). Protein extracts will be diluted with 1% TFA and bound to the C8 spin column. After washing with 1%TFA, protein will be eluted from the column in at least four steps ranging from 5 to 50% acetonitrile, dried, resuspended in matrix and analyzed by MALDI-TOF MS. If neither of these methods sufficiently reduces the sample complexity, then both affinity procedures will be performed in series. Cleanup steps and analyses will be automated as much as possible.

D3-SA#5: Specific Aim #5. To evaluate the applicability of ISMA technology to real-world situations by deploying prototypes of the device at a highly contaminated and well-characterized VOC-containing Superfund site.

The project will conclude with a field test of the ISMA sampler at the Building 834 Operable Unit at Site 300. Samplers will be configured with three types of compartments: glass wool plugs only, glass wool plugs containing 10% agar by volume, and glass wool plugs containing 10% agar by volume supplemented with 10g/kg of ^{13}C-labeled tetraalkoxysilane (custom-synthesized tetrabutoxysilane or tetrakis(2-ethylbutoxy)silane). ISMA samplers will be prepared in the laboratory, placed on ice and transported to the field. Prior to deployment, the compartments of both samplers will be flooded with sterilized distilled water in order to replace air. Samplers will be lowered into monitoring wells W-834-D3 and W-834-T5 to depths equivalent to half height of the well screen (25 ft and 70 ft, respectively). Initially, the device will be operated in flow-through mode for 5-14 days and retrieved. Back at the surface, the device will be drained, placed on ice and shipped overnight to the laboratory for analysis. Several sampling events are planned. The PI will train the LLNL personnel, conduct the first deployment of the sampler, and will observe the second sampling event. Following this, the trained LLNL staff (Mrs. Gregory and Madrid) will independently conduct any additional sampling as needed.

The ISMA samplers will be analyzed as described above using the molecular-genetic analysis approach. The content of replicate compartments may be combined in order to increase the amount of DNA extracted. The work will focus primarily on the extraction and analysis of the ^{13}C- labeled heavier DNA fraction. Field sampling will be repeated and modified as needed in order to maximize the growth of microorganisms metabolizing labeled tetraalkoxysilane-related compounds. Obtained samples will be screened for the presence of heavy DNA and sampling will be repeated as necessary. Heavy DNA from a successful field trial will then be analyzed completely including sequencing of DGGE bands and comparative sequence analysis.

Comparative sequence analysis. Obtained sequences (>500 bp) will be analyzed using BLAST (1) and added, together with most important BLAST hits, to an alignment of about 5,300 homologous bacterial 16S rRNA primary structures (35) by using the aligning tool of the ARB software package (http://mikro.biologie.tu-muenchen.de). Sequences will be checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (35). Potential chimeras will be eliminated and phylogenetic trees constructed.

The work detailed in Section D3-SA#1 will produce two functional prototypes of the ISMA sampler. Computer modeling results obtained using the final design parameters will indicate the maximum depth to which the devices can be lowered safely for groundwater monitoring. Statistical analysis of the two datasets obtained from the experiments described in Section D3-SA#2 will yield information on (a) the variance of feed rate as a function of microcosm capillary location within the ISMA, (b) the efficiency of cell capture for each of the five microorganisms which differ in size and surface characteristics, (c) the microbial accumulation effect one may achieve in field situations via *in situ* filtration of defined groundwater volumes, (d) the effectiveness of the device to selectively enrich specific microorganisms of interest from complex microbial mixed cultures (this concentration factor is critical for successful MALDI-TOF MS analysis). Results from the laboratory SIP experiments detailed in Section D3-SA#3 will inform about the potential usefulness of incorporating stable isotopes for selectively labeling microorganisms *in situ* using the ISMA. The work described in Section D3-SA#4 will produce proof-of-concept data on (a) the use of MALDI-TOF MS for automated ISMA analysis for identification of bacteria, (b) the type and nature of biomarkers occurring in the mass spectra of the five model microorganisms, (c) the minimal number of target bacteria required for detection, and (d) the possibility of detecting functional gene products by MALDI-TOF MS using readily automatable sample processing techniques. The field experiments described in Section D3-SA#5 will (a) provide proof-of-concept data on the performance of the sampler in real-world situations, (b) provide a supplemental dataset on the microbial community of the study location that can be evaluated in the context of previous studies, and (c) potentially reveal which of the organisms detectable at the site are involved in the transformation of tetraalkoxysilanes, the drivers of reductive dechlorination at the site.

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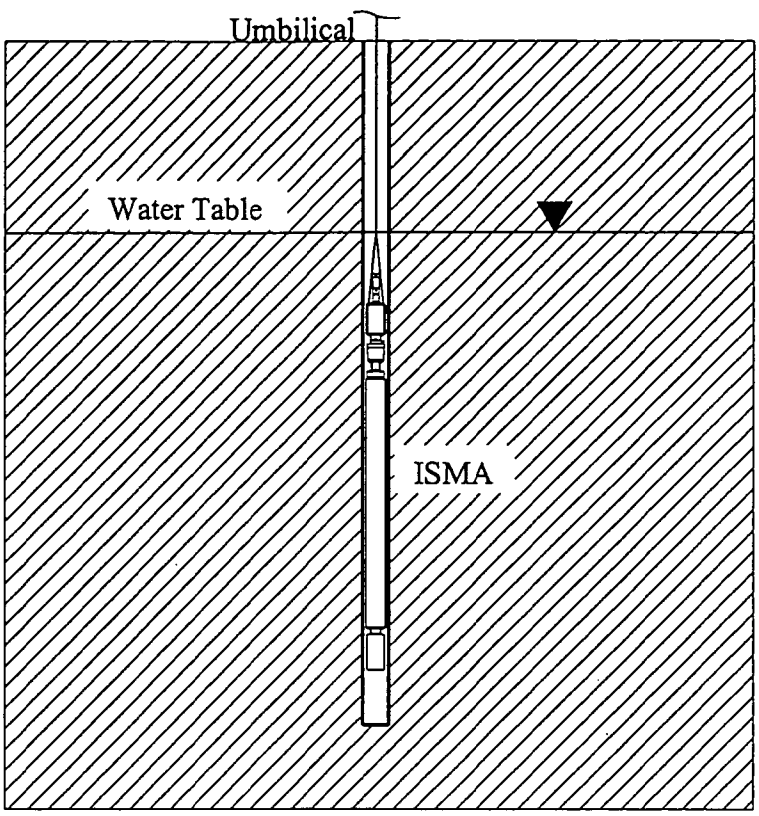
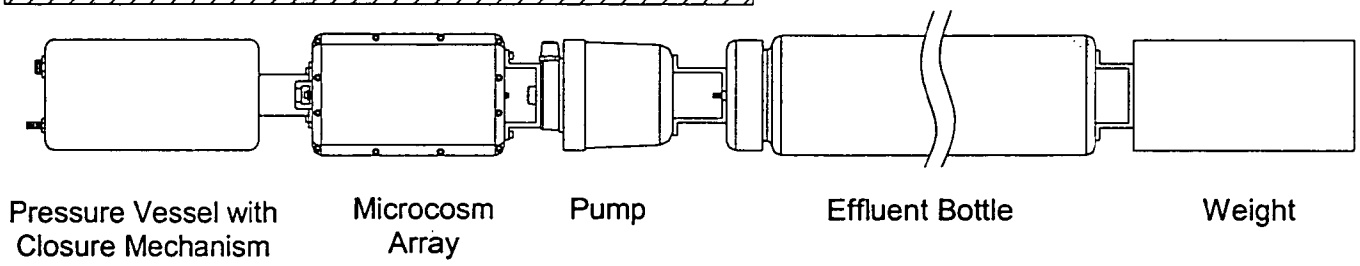


Figure 4.
Schematic showing the *in situ* microcosm array (ISMA) suspended in a standard 100-mm diameter groundwater monitoring well. The device is supported from the surface via an umbilical, that holds it in place and provides power and vacuum for actuation of the integrated closure mechanism and pump.

Figure 5.
In situ microcosm array (ISMA) system components.



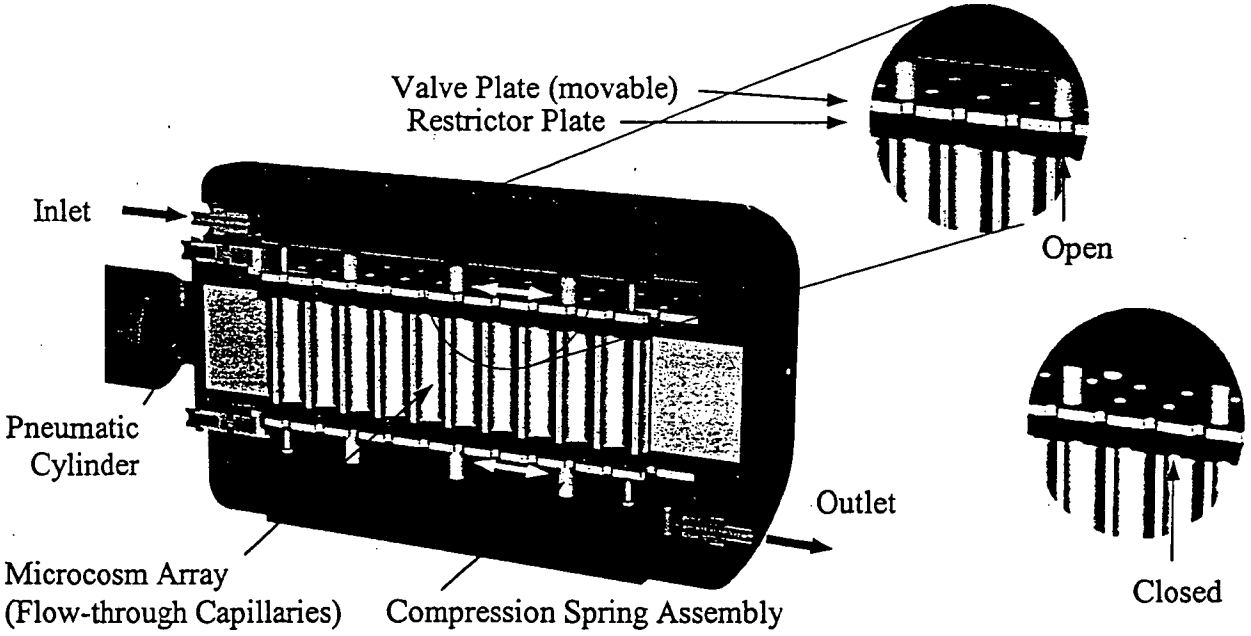


Figure 6. Cutaway view of the closure mechanism and the microcosm array (see Section D for details).

mechanism sealing the array. After a sufficient incubation period, the device is removed from the well and taken to a lab where the array is extracted and samples are analyzed.

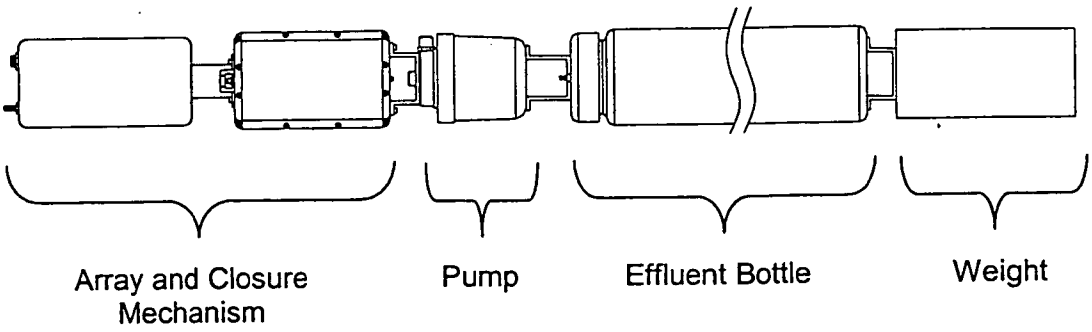


Fig. 2 ISMA system components.

Array and closure mechanism description

The ISMA utilizes a custom 96-well microtiter array and closure mechanism to remotely seal the microvessels after a sufficient sample has been collected; see Fig. 3. The microvessels are 7.5 mm diameter and 25 mm deep yielding a working volume of 1100 µl. As shown in the figure, flow is injected into the mechanism via a single inlet filling the upper cavity above the valve plate. In the open configuration shown in the figure, the holes in the upper and lower valve plates are aligned with holes in the underlying restrictor plates allowing fluid to pass through the array. Flow exits from the lower cavity via a single outlet. To seal the array, the valve plates are translated horizontally blocking the holes in the restrictor plate and cutting off the flow. The valve plates are translated using a pneumatic cylinder. A compressive force is applied to the valve plates using springs to facilitate a tight seal between the valve and restrictor plates.

Operating depth

The operational depth of the device is limited primarily by hydrostatic stress. Based on conservative estimates, the device should function normally in depths up to 10 m. More detailed analyses and tests will have to be performed to determine the true depth limit.

ISMA Closure Mechanism

The In-Situ Microcosm Array (ISMA) system proposed will be used to remotely extract and culture environmental samples for bioremediation studies. The ISMA is designed to be suspended into a 100 mm (4") diameter well at depths up to 100 m (~300 ft.) and submerged up to 10 m (~30 ft.) below the water; see Fig. 1. The system collects ground water samples over a period of time and then seals creating an anaerobic environment suitable for culturing ? (Rolf) microorganisms.

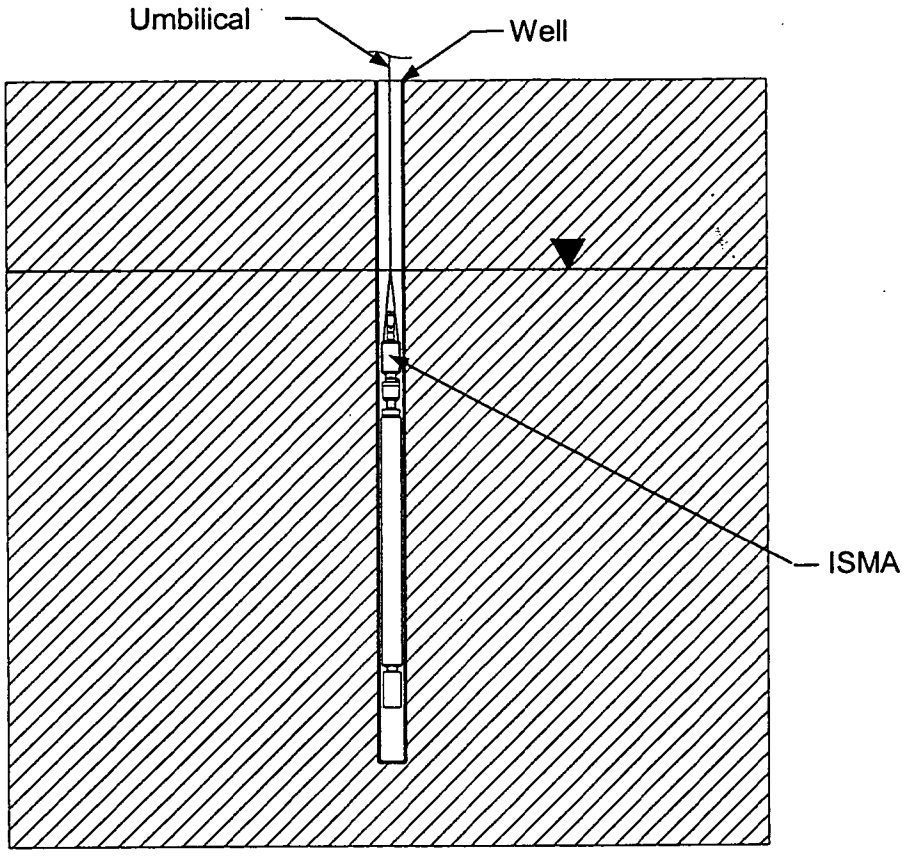


Fig. 1 Schematic showing the ISMA suspended in a standard 100 mm diameter well. The device is supported from the surface via an umbilical, which supports the device and provides power and telemetry to the surface.

System level description

The ISMA system is shown in Fig. 2. It consists of a pump, a 96-well microtiter array, a closure mechanism, an effluent bottle, and a weight. The system functions as follows. The device is lowered into the well with the array sealed. The closure mechanism is opened via a pneumatic cylinder and ground water is transferred to the microtiter array from the pump. Ground water flows through the array exiting into the effluent bottle. Microorganisms are trapped in the webbing placed inside the array microvessels as the as fluid flows through the device. When the effluent bottle is full, a float trips power to the pump and actuates the pneumatic closure

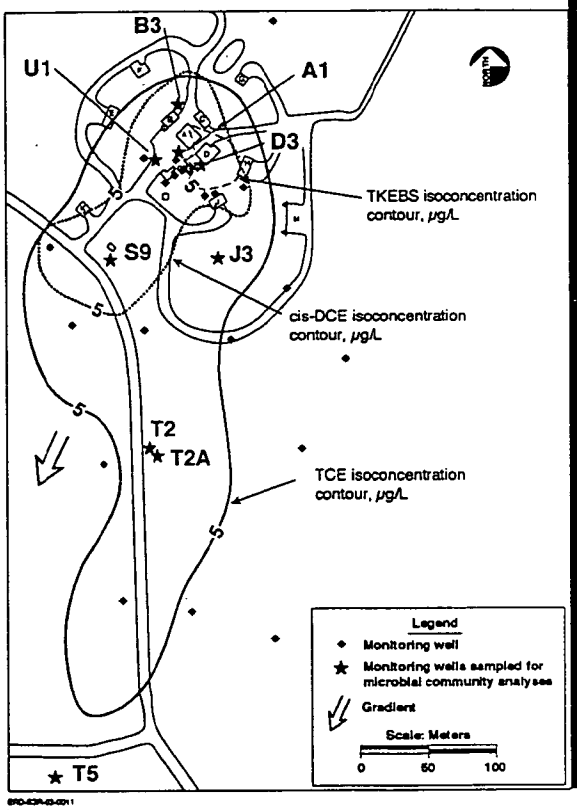


Figure 2.
Field site map indicating the locations of monitoring wells at the Building 834 Operable Unit at LLNL Site 300, CA. Five-ppb-contours show the extent of groundwater contamination caused by spillage of trichloroethene (TCE) and tetrakis(2-ethylbutoxy)silane (TKEBS). The contour for *cis*-1,2-dichloroethene (*cis*-1,2-DCE) indicates the presence of intrinsic reductive dechlorination activity at the site.

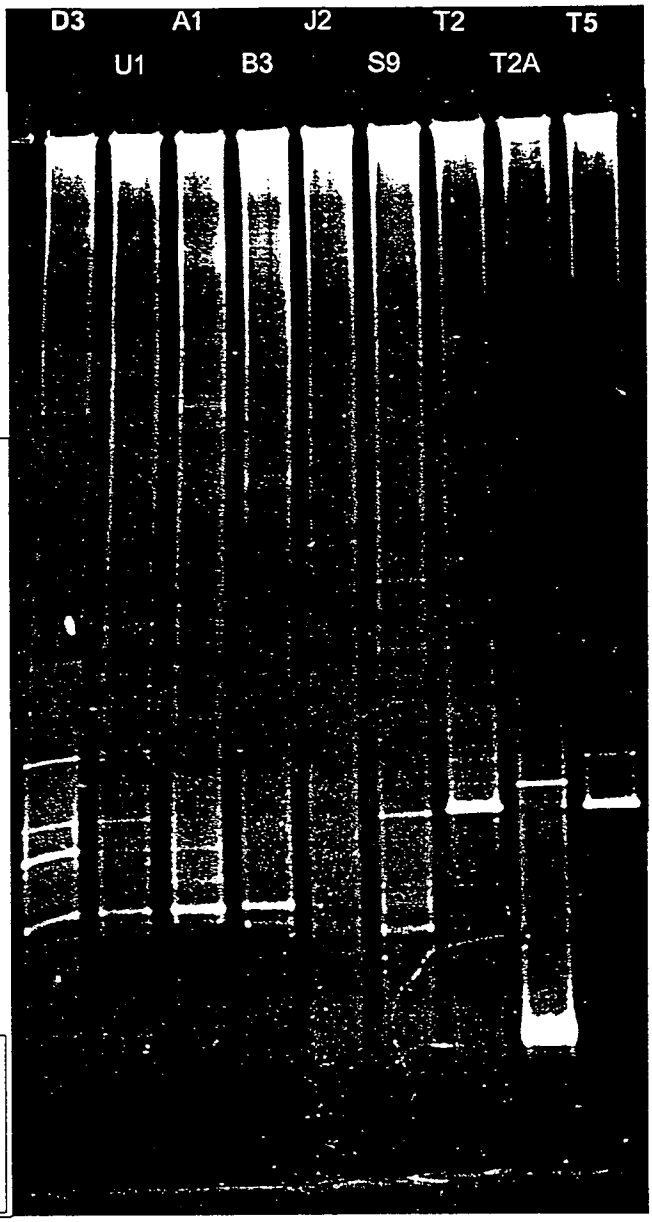


Figure 3.
Composite digital image of an ethidium bromide-stained DGGE gel (35-55% denaturant) separating the bacterial DNA fragments coding for 16S rRNA. Using the primers GC-GM5f and 907r, fragments were amplified directly from DNA extracted from the following groundwater monitoring wells at LLNL Site 300: W834-D3, -U1, -A1, -B3, -J2, -S9, -T2, -T2A, and -T5.

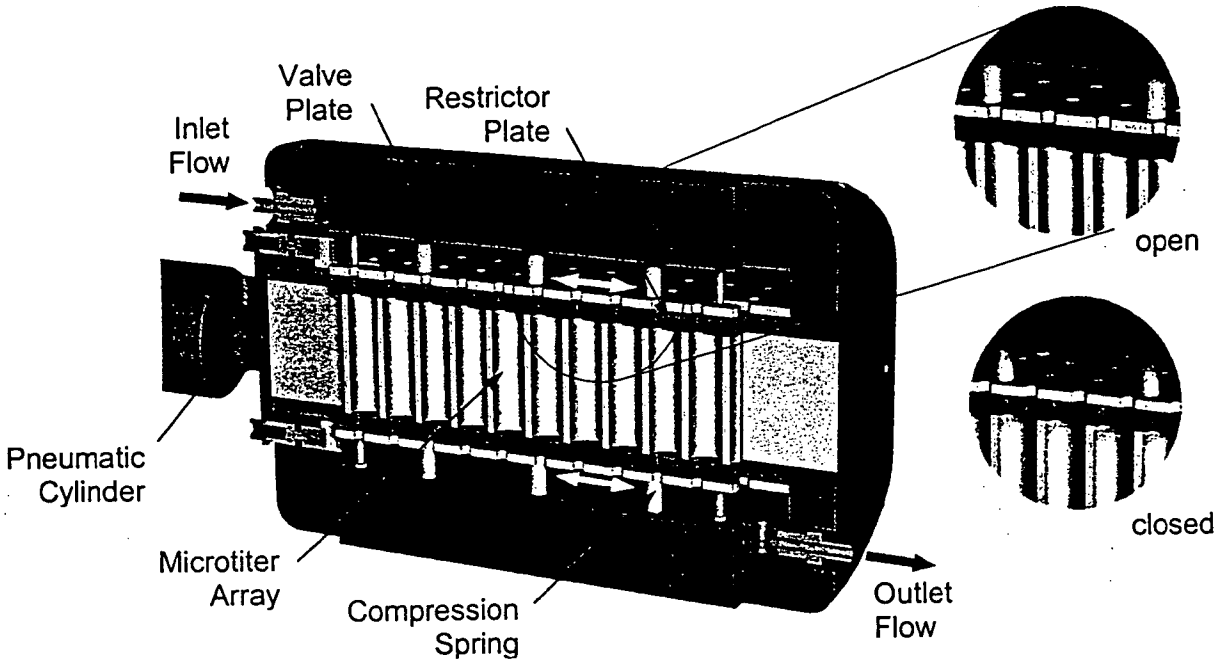


Fig. 3 Cutaway view of the closure mechanism and microtiter array. Detailed views show the alignment of the valve and restrictor plates in the open and closed configurations.

Mass

The dry mass of the ISMA system is 7.2 kg (15 lbf). The dry mass on a subsystem basis is as follows: array and closure mechanism – 1.7 kg (3.8 lbf); effluent bottle – 1 kg (2.2 lbf); pump – 1.8 kg (4 lbf); weight – 2.7 kg (6 lbf). When the effluent bottle is full the mass increases by 5 kg bring the resulting in a total wet mass of 12.2 kg for the system.

Closure mechanism actuation

The closure mechanism is actuated using a pneumatic cylinder. Pressure is supplied to the cylinder locally using a small pre-charged pressure vessel. In order to keep the unit compact, the pneumatic cylinder, control valves, and position sensors are encased in the pressure vessel. Prior to deployment into the well, the vessel is charged. A low voltage electrical feed from the surface is used to switch the state of the control valves; thus opening/closing microtiter valve plates. Signals from the position sensors on the cylinder are fed to the surface indicating the state of the device. Bleed off air from the cylinder is vented above the water table via a tube in the umbilical.

1. **Abstract of the Invention** [Briefly describe the invention]

A new tool has been devised allowing for the in situ environmental monitoring of (i) biodiversity, (ii) microbial metabolic activity of pure and mixed cultures, (iii) the behavior, survival and fate of native and introduced microorganisms in natural environments, and (iv) the flow of energy, carbon atoms and other elements and molecules in native microbial communities. In addition, the device can be applied for (v) studying abiotic reactions of chemicals in solution and on surfaces, (vi) the discovery of novel natural products, (vii) bioprospecting for novel microorganisms, and (viii) the cultivation of microorganisms that cannot successfully be grown or maintained in the laboratory environment. The tool—in situ microcosm array—is made from an inert material and contains a large number of compartments (tens to thousands) designed for capturing and cultivating microorganisms in their natural environment and for determining their biochemical potential and activities in situ. The compartments of the tool serve as biochemical test vessels. Each compartment contains test substances (organic or inorganic compounds delivered in the dissolved, solid or gaseous phase) that may be labeled with isotopes for uptake by, or binding to, metabolically active microorganisms. The tool is used by exposing the individual test compartments of the device to the matrix of interest (e.g., submerging the device in groundwater or seawater) and by incubating it in situ prior to analysis. Integrated pumps and closure mechanisms facilitate controlled flux of chemicals and the test matrix (e.g., groundwater or seawater) through the device thereby allowing for operation in flow-through mode, batch mode, open-system mode or a combination of the above. Typical test compounds are environmental pollutants, electron donor/acceptor compounds, chemical indicators reporting on the presence of a desired biochemical/metabolic function, growth factors or biochemical inhibitors favoring the growth of specific microorganisms or microbial communities, as well as microbial carbon sources and energy sources. The device may also be amended with bacteria/spores/ viruses and protozoa to determine their survival in the environment and to measure any change they may cause, including ecological effects that are of interest for ecological risk assessment. Following incubation in the test environment, the tool is analyzed for biochemical marker compounds (*e.g.*, metabolites, 16S rDNA, ^{13}C-labeled DNA, ribosomal proteins, etc.) that are characteristic for the trapped indigenous microorganisms, using physical, chemical, biological, genomic and/or proteomic approaches. Analysis of isotope-enriched markers allows for identifying metabolically active microorganisms. Additional analyses can be performed on the environmental sample and/or on the inner surfaces of the tool in order to determine biofilm composition and microbially-induced change (chemical or physical) within the test system or within the fluids that were passed through them. Collecting all of the effluent of the various test vessels combined or individually allows for performing a complete mass balance on both chemicals and microorganisms. The above analyses provide both a picture of the microbial community and a corresponding rate of chemical, biochemical and/or physical change. Computational analysis of the multiple community profiles and corresponding rates of change— by e.g., subtractive profiling—can be used to link observed functions to specific microbial community members. Use of inhibitors for selected subpopulations aids in this task. This technology is novel in that it combines automated biochemical in situ screening, use of isotopes (stable and/or radioactive), in situ sampling and incubation, as well as laboratory-culture-independent microbial community analysis. It can serve to link observed reactions/activities to distinct members of complex microbial communities. It can reveal the presence of novel microorganisms, biomolecules, metabolic functions and cell signaling while recording the chemical composition of the target environment at the same time. Therefore, it can be applied to the environmental monitoring of cleanup sites, biodiversity prospecting studies, and ecological risk assessment studies. When used to forecast reaction rates in altered environments, systematic biases introduced by the measuring technique may be accounted for by standardizing the analysis, by including test compounds and living microorganisms for normalization and direct comparison of test results, and by using algorithms that correct measured rates for biases via interpretation of databases containing pairs of predicted and actual rates measured following environmental manipulations (e.g., nutrient and oxygen addition). The device also may be applied in bioaugmentation studies and in assessing the environmental survival and impact of native microorganisms and, more importantly, introduced non-native microorganisms and genetically modified organisms. In addition, it can be deployed in deep-sea environments for bioprospecting and ecological studies.

2. Problem Solved [Describe the problem solved by this invention]

The new tool and analysis strategy allows one to determine the microbial community structure of complex environmental mixed cultures, to link an observed chemical, biochemical and/or physical change to a particular microorganism, to study microbial interactions, and to culture and study previously uncultivated microorganisms in pure culture and during interaction with their natural environment. Due to the incubation of the tool in situ, rates and metabolic activities determined with the device are expected to closely mirror actual actions currently occurring or potentially occurring *in situ*. The use of isotopes in conjunction with molecular-genetic and/or proteomic analysis techniques allows one to distinguish dead and dormant microorganisms from metabolically active ones (only viable cells will incorporate isotope labels into biomarkers). Parallel testing of effects caused by various environmental parameters (*e.g.*, type and concentration of added nutrients/mixtures/microorganisms) allows one to deduce which of the metabolically active microorganisms are responsible for an observed change. This has important implications for the design and monitoring of bioremediation strategies, e.g. bioimmobilizaiton of uranium by bacteria, or the dechlorination of toxic chloroethenes, etc. Taken together, these characteristics of the new technology provide a hitherto unattained level of discriminatory power that will enable one to selectively enrich for, culture and identify novel microorganisms and microbial functions. This is of great importance for the cleanup (bioremediation) of contaminated sites and for the biological prospecting for novel microorganisms, biomolecules, drugs and metabolic processes. Furthermore, the technology can be used for the in situ cultivation of microorganisms that do not grow in the laboratory, and for assessing the survival and metabolic activity of foreign species in natural environments, which is of importance to public health.

3. Novelty [Identify those elements of the invention that are new when compared to the current state of the art]

The tool and analysis strategy are novel because they allow for the first time the cultivation and comprehensive biochemical characterization of microorganisms in their natural environments. The technology is novel in that is combines in a non-obvious fashion the following tools/approaches: solid-phase sampling techniques, in situ enrichment and biochemical screening, use of electron donor/acceptor pairs, isotope labeling and massive parallel screening with automated analysis. The technology is novel in that it provides data for hundreds or even thousands of hypothetical environmental scenarios, thereby allowing one to determine quickly and in an automated fashion the likely rates of environmental change induced by these perturbations. The strategy is novel in that it makes use of in situ microcosm arrays in conjunction with culture-independent microbial community analysis to obtain a comprehensive picture of microbial communities. It is suitable for linking specific microbes to observed reactions by using computer-assisted subtractive profiling techniques. It is fully compatible with existing robotic systems thereby allowing for rapid and fully automated analysis using chemical, physical, biological, genomic and—more importantly—proteomic analysis techniques. The proposed inclusion into the in situ microcosm array sampler of miniaturized pumps, closure mechanisms, semi-permeable membranes and filters is new as it will allow one to first inoculate and then incubate the device in the environment without removing (and potentially harming) the resident microbes from their natural environment. The device can be equipped with microfluidic systems allowing for delivery of small volumes and defined quantities of microorganisms to the test chamber prior to physical and/or chemical containment of the captured specimens via barriers that are either non-permeable, semi-permeable or completely permeable for chemical compounds; this aspect will allow one to culture uncultivated or "non-culturable" bacteria to numbers sufficiently large to perform biochemical characterization and identification. The technology is suitable for determining the rate of protozoan grazing in situ. The device also allows one to determine how non-native microorganisms will cope in natural environments when confronted with physical, biological and/or chemical stressors. For this application, test organisms will be inoculated into the device prior to its deployment. Semi-permeable membranes can allow the introduced species to come into contact with the target environment while staying contained in the device. Inoculation of some of the test chambers with known quantities of test microorganisms also can assist in determining the toxicity of a natural environment and in normalizing assessment data for direct comparison of geographically distinct environments.

4. Detailed Description of the invention:

On a separate page(s), attach a detailed description of how to make and use the invention. The description must contain sufficient detail so that one skilled in the same discipline could reproduce the invention. Include the following as necessary:

Please refer to the attached two grant proposals and one abstract, taken from a letter of intent sent in response to a request for proposals.

1- data pertaining to the invention;
2- drawings or photographs illustrating the invention;
3- structural formulae if a chemical;
4- procedural steps if a process
5- a description of any prototype or working model;

In general, a manuscript that has been prepared for submission to a journal will satisfy this requirement.

Configurations of the in situ microcosm array (ISMA) for bioremediation and bioprospecting in addition to those mentioned in the attached research proposals.

In addition to the details provided in the attached research proposals, the device can be equipped/used as stated below:

Sorbent materials for chemical analysis. Effluent from the individual flow-through microcosms will be passed through a sorbent material (e.g., chromatographic columns, C-18 solid-phase-extraction plates [Spec manufactured by Varian], ion exchange cartridges, disk filters, membranes or other) to sorb and capture selected chemicals/specimens of interest. Following retrieval of the device, chemicals/specimens collected on the sorbent can be removed and analyzed. This allows one to conduct a complete mass balance on microorganisms and chemicals entering and leaving the device. For this purpose, sorbent arrays can be located downstream and/or upstream of the microcosm array.

Individual collection vessels. The attached drawings shows a single receptacle for the combined effluent of all flow-through microcosms. Alternatively, the effluent of the individual microcosms may be collected separately. Thus, the single effluent bottle shown in the attached research proposal may be replaced by a manifold connected to hundreds or thousands of small bladders that can capture the effluent from each individual microcosm thereby providing an absolute mass balance on all materials that passed through each of the microcosms.

Standardized microcosms. Each ISMA can contain a number of "standardized microcosms." The latter are flow-through microcosms containing a known quantity of well-defined microorganisms and varying amounts of test compound(s) (none to high concentrations). Standardized microcosms will provide a measure of the toxicity of the test environment. In addition, analysis of the survival and growth of these microorganisms and their metabolic activities under the respective conditions will allow one to normalize test results for ISMA samplers deployed in different locations and at different points in time.

Integration of filters in selected microcosms. Selected microcosms will be equipped with a filter (placed at the inlet or further upstream of the flow-through microcosm). Filters will allow one to selectively exclude certain micro-

organisms from entering a flow-through cell. For example, exclusion of protozoans will allow one to determine the rate of protozoan grazing by comparing the results of two sets of microcosms that were identical except for the presence of the filter in one set of the systems. Similarly, one may exclude larger bacteria using a particular pore-size filter to selectively enrich for small bacteria (micro- and nanobacteria).

Modifiers. Selected flow-through microcosms will be equipped with chemical inhibitors, inducers and similar chemical modifiers. This will allow one to selectively induce the expression of proteins and metabolic functions of interest in captured microorganisms. In addition, chemical modifiers may be used to selectively suppress subpopulations within the device. For example, antibiotics can be included to suppress growth of fungi, certain bacteria and protozoa. Similarly, inhibitors can be included to prevent the growth and activity of microbial subpopulations; for example, sulfate reducing bacteria and methanogens may be selectively inhibited using sodium molybdate and BES, respectively. Additional selective inhibitors exist that inhibit other subpopulations.

Test compound delivery system. Agar is only one of many substances to be used as a substratum for microbial colonization and as a medium for continuous release of test compounds. Alternative materials include gellan gum with or without CaCl (Jansen et al. 2002), and other inert materials such as glass or plastic that can be molded to form columns, porous networks, beads, etc. Test compounds may also be presented to microorganisms within the microcosms using solids (crystals) and coatings of poorly water-soluble compounds (nonaqueous phase liquids). Gases may be delivered to the system using any of the following techniques: in situ generation of the desired dissolved gaseous species; adsorbed gases; semi-permeable membrane vesicles filled with gasses of interest (passive gas delivery); or active gas delivery using miniaturized pressurized gas bottles.

Pumps. The test medium (e.g., groundwater or seawater) may be delivered by a single pump using conventional pump systems such as centrifugal pumps, rotary pumps, piston pumps, syringe pumps (twin configuration; one syringe delivers while the other is being filled), peristaltic pumps and/or bladder pumps. Alternatively, multiple pumps may be used to achieve similar flow rates in all systems regardless of pressure buildup that may occur as a result of in-line filters, physical clogging and microbial growth. These pump arrays may use any of the above pump mechanisms or equivalent others.

Microfluidics and multiple arrays. Some applications may benefit from the use of microfluidics and operation of multiple arrays in parallel or in series. For example, for bioprospecting studies the number of test compartments may be as high as several thousand per microcosm array. These miniaturized systems will be fed with test medium (groundwater, seawater, etc.) using microfluidic systems that minimize dead volume within the device and allow for delivery of (sub-)microliter quantities of test medium to the individual microcosms. This configuration will be ideal for bioprospecting studies to cultivated otherwise "non-culturable" microorganisms. Following delivery of a small volume of test medium to a microcosm, the valve plates may move into an intermediate position thereby placing a semi-permeable membrane at the entrance and exit of a microcosm. Thus, individual microbial cells may be trapped randomly in a single microcosm. These may then be incubated in a flow-through mode that allows chemicals to enter and exit the test volume while microorganisms are prevented from moving in or out of the test vessel. In this way, confined microorganisms may be cultured while still being in "chemical communication" and interacting with other microbial community members. Cell signaling and other chemical interactions occurring only in situ are known to be essential for certain microorganisms to proliferate. Operating thousands of microcosms in one array and several of these arrays in parallel or in series will facilitate high throughput screening of large numbers of organisms under diverse test conditions.

Adaptation of the device for deep-sea exploration. Using the configuration and materials shown in the attached drawing, the device is estimated to withstand depth of 100 meter and below. Alternative materials such as the use of stainless steel with or without polymer coating will make the device suitable for deep-sea deployment. The umbilical shown in the attached figure may be replaced with a remote and/or programmable control mechanism.

5. **Workable Extent/Scope** [Describe the future course of related work, and possible variations of the present invention in terms of the broadest scope expected to be operable; if a ***compound,*** describe substitutions, breadth of substituents, derivatives, salts etc., if ***DNA or other biological material,*** describe modifications that are expected to be operable, if a ***machine or device,*** describe operational parameters of the device or a component thereof, including alternative structures for performing the various functions of the machine or device]

The proposed technology has a broad workable extent. Microfluidics, filters of varying sizes, semi-permeable membranes and alternative closure mechanisms may be integrated into the sampler to separate in time the inoculation of the device from the incubation period that allows chemical change to take place within the sampler. Optical and/or electrical detection systems may be incorporated in microfluidic configurations to seal individual microcosms as soon as a single cell has been delivered to the microcosms, thereby greatly increasing the success rate of isolating novel microorganisms. Proteomic approaches may be used for rapid and fully automated analysis (e.g., matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and protein sequencing of enzymatic digests using tandem mass spectrometry (MS/MS). Central facilities may be used for analyzing samplers deployed in situ. This will allow for automated analysis and for a high degree of standardization. Standardized analysis in turn will dramatically improve measurement precision and will allow one to determine the systematic biases of the technique (due to "bottle effects") that may limit measurement accuracy; once identified, these biases can be accounted and corrected for thus enabling one to predict with high accuracy and precision the environmental change to be observed following engineering interventions. For bioremediation purposes, this would entail the development of databases that record predicted biotransformation rates and rates actually observed in situ. The format of the tool allows for automated analysis. Speed and ease of analysis may be achieved by replacing molecular-genetic analyses with other more convenient measurement techniques suitable for discerning isotope distributions (e.g., use of MALDI-TOF MS and bioinformatics database searches for automated microorganism identification). Sample processing using commercially available robotics (Amersham Biosciences robotics) and tools for rapid sample cleanup and processing (e.g., Gyrolab MALDI SP1 etc.) in conjunction with enzymatic digestion steps (e.g., trypsin digestion).

The device also may be adapted for studying the fate of either beneficial or hazardous biological agents in natural environments. This work would require the device to be modified to reflect as closely as possible within each test compartment the physical/chemical/biological environment of interest (e.g., flow-through cells equipped with local sediment etc.). Again, the device would be equipped with a semi-permeable barrier allowing for interaction of the test species with the environment without allowing for its release.

6. **References** [Please list the closest and most relevant journal citations, patents, general knowledge or other public information related to the invention]

Geyer, R., A. D. Peacock, Y.-J. Chang, Y.-D. Gan, and D. C. White. 2002. Presented at the 2002 NABIR PI Conference, Arlie, VA. *Down-Well Microcosm "Bug Traps" and Subsurface Sediments for Rapid expanded-Lipid-Biomarker Analysis and DNA Recovery for Monitoring Bioremediation Microbial-Community Ecology within Samples from Uranium-Contaminated Sites.* In *2002 NABIR PI Conference.* 2002. Arlie, VA.

Nayar, S., B. P. L. Goh, L. M. Chou, and S. Reddy. 2003. In situ microcosms to study the impact of heavy metals resuspended by dredging on periphyton in a tropical estuary. Aquatic Toxicology 64:293-306.

Zengler, K., G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller. 2003. Cultivating the uncultured. PNAS.

Short, J. M., and M. Keller. 2001. U.S. Patent 6,174,673

Connon, S. A., and S. J. Giovannoni. 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates.

☐ No references available at this time.

Integration Of Real-Time Sensing Technology Into The ISMA Device

The drawing (file name: sensor_layout.jpg) shows the layout for integration of real-time sensing technology into the ISMA device. Each of the individual test compartments or capillaries is equipped with an orifice (ranging in diameter from 100 to 1000 micrometers) that can accommodate one or multiple protruding sensor heads or multi-parameter sensor heads. Signal transmission is achieved via one of the following mechanisms (1) wiring, (2) flexible optical fibers, or (3) another suitable technique allowing for transmission of sensing information from each compartment to the exterior of the device. As shown in the drawing, individual sensing channels are first running in parallel and, on their way to the exterior of the device, form successively larger growing arteries or transmission channel bundles. In the drawing, all 96 (and possibly more) channels are exiting jointly in a central single location. Other configurations not shown here are possible and may be chosen to not exceed bending limits imposed by optical fibers of limited flexibility. The latter situation will require multiple openings and channels, leading to individual compartments in a straight or curved fashion suitable for accommodating more rigid materials.

Starting And Stopping Reactions Within The Device

A layout identical or similar to the above channel configuration may also be used to inject test chemicals into the device at predetermined times or when desirable conditions have been reached as signaled by the integrated sensors. Reactions can be executed as follows: first, the valve plates nearest to the effluent container is being translated to allow for partial displacement of capillary content; next, chemicals contained in a reservoir or concentrated in the transmission channels are delivered into one, multiple or all compartment by means of a pressure pulls generated using (1) the integrated pressure container (gas driven), (2) an additional pressure container, or (3) another delivery mode such as an integrated pump. Following delivery of the test chemical, substance or organism, valve plates may stay open for flow-through operation or may be closed for batch operation. The orifice leading to the capillary lumen is equipped with a miniaturized check valve that allows unidirectional flow during delivery of the injection pulse while preventing backflow of liquids into the transmission channels before, during and after incubation. This mode of delivery can be exploited to deliver (1) sensitive materials that are susceptible to degradation during initial submersion and deployment of the device, (2) microorganisms that do not tolerate initial conditions in the device, e.g., obligate anaerobic bacteria whose survival and cultivation requires anoxic conditions, (3) biological agents that are in a dormant state such as microbial spores (e.g., Bacillus anthracis), and (4) "trigger compounds" that either initiate or end a given process; for example, excessive build-up of microorganisms (biofouling) can cause a reduced flow rate. Flow sensors sensing the undesirable change will trigger the release of a sodium azide solution (or another agent) to the compartment that then will bring ongoing reactions to a complete stop. This process will allow one to take a snapshot of chemical, biological or physical conditions within the device at discrete time intervals in single, multiple, or all compartments. It also can facilitate the delivery of labeled marker molecules into the device upon reaching of desired conditions. This latter option is useful

when using expensive isotopes and other labels of which only the absolute minimum quantity should be used for economical reasons or due to environmental health and safety consideration.

Integrated Control Mechanism Signal

Transmission channels may be configured with triggered or automated feedback loops that translate a sensing event into a mechanical maneuver within the device, e.g., closing of valve plates or targeted heat sterilization via (1) electrical heating or (2) chemical heating via delivery of a reactive compound or a combination of chemicals triggering exothermic reactions.

Wireless Signal Transmission

For operation in remote or extreme environment, the ISMA device can be equipped with a small battery and transmitter (located externally or integrated into the device) to relay signals from the device to the surface or a conveniently placed control module. Similarly, signal transmission from the control station to the device can be achieved by integrating receivers into the device either externally or internally. Power sources may be recharged using existing redox gradients or other renewable energy sources. There may be a need for signal amplification, which can be accomplished by amplifiers placed along the transmission pathway, to maintain signal strength when bridging large distances or challenging conditions, such as signaling from deep-sea environments to sea level.

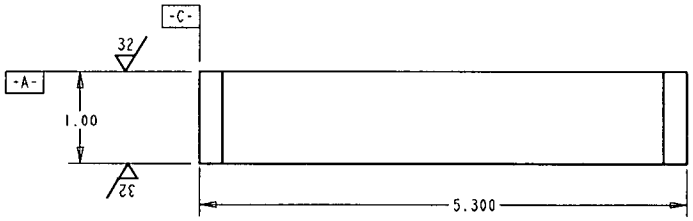
Materials Compatibility And Design Modifications For Diverse Environments

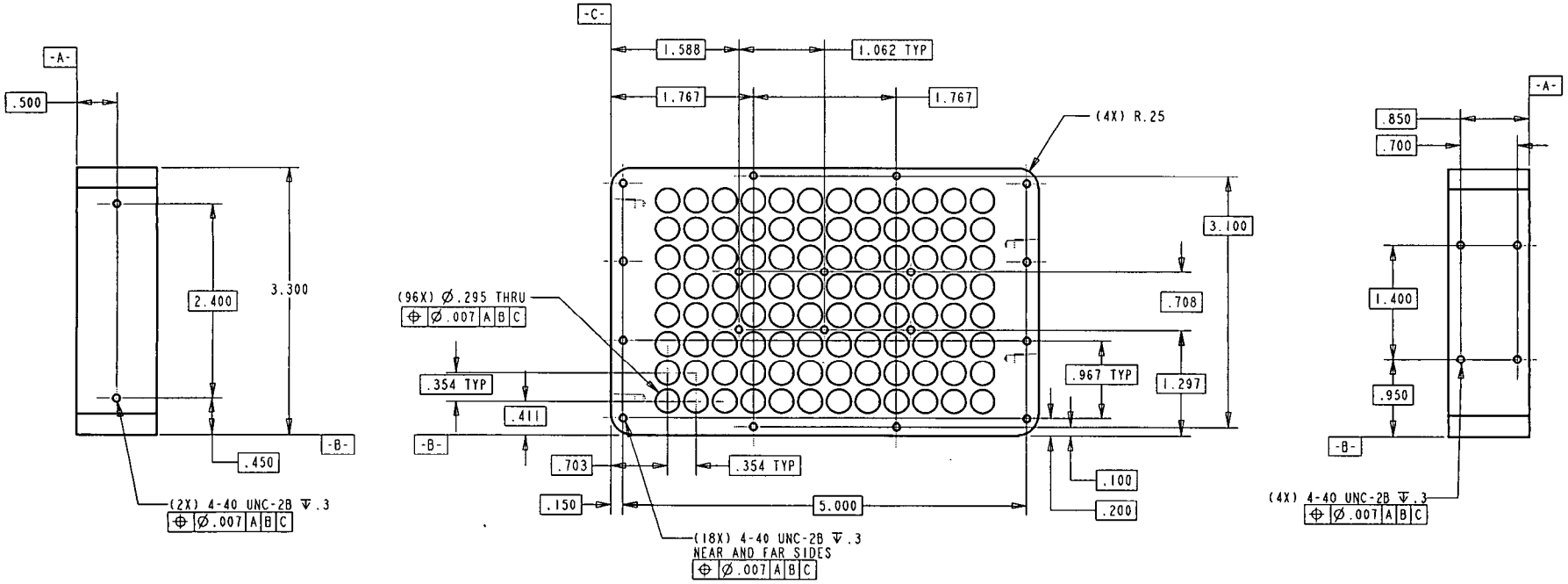
Materials used in the manufacturing of system components can vary and will be selected based on the desired application in order to withstand chemical, biological, or physical stress or a combination thereof. For example, for deployment in deep sea environments, system components may primarily consist of metals and Teflon-coated metals that can withstand extreme pressures and temperatures encountered in hot smokers and thermal vents. Similarly, the dimensions of the prototype shown in the drawings may be either dramatically increased (structurally reinforced, larger apparatus) to allow for application in conditions of extreme pressure, temperature, etc., or may be dramatically decreased (micro-electro-mechanical systems; MEMS) to generate nanotechnology devices suitable for use in human and animal testing of bodily fluids. In these applications, the device may take the form of a flexible pad worn externally. Alternatively, it may be used to study the chemistry and biology within living macro-organisms (plants, animals, humans) by serving as an implant that may be delivered surgically, be swallowing or another delivery mode.

REV.	DESCRIPTION	DATE	APPROVAL

NOTES:

1. DEBUR ALL SHARP EDGES





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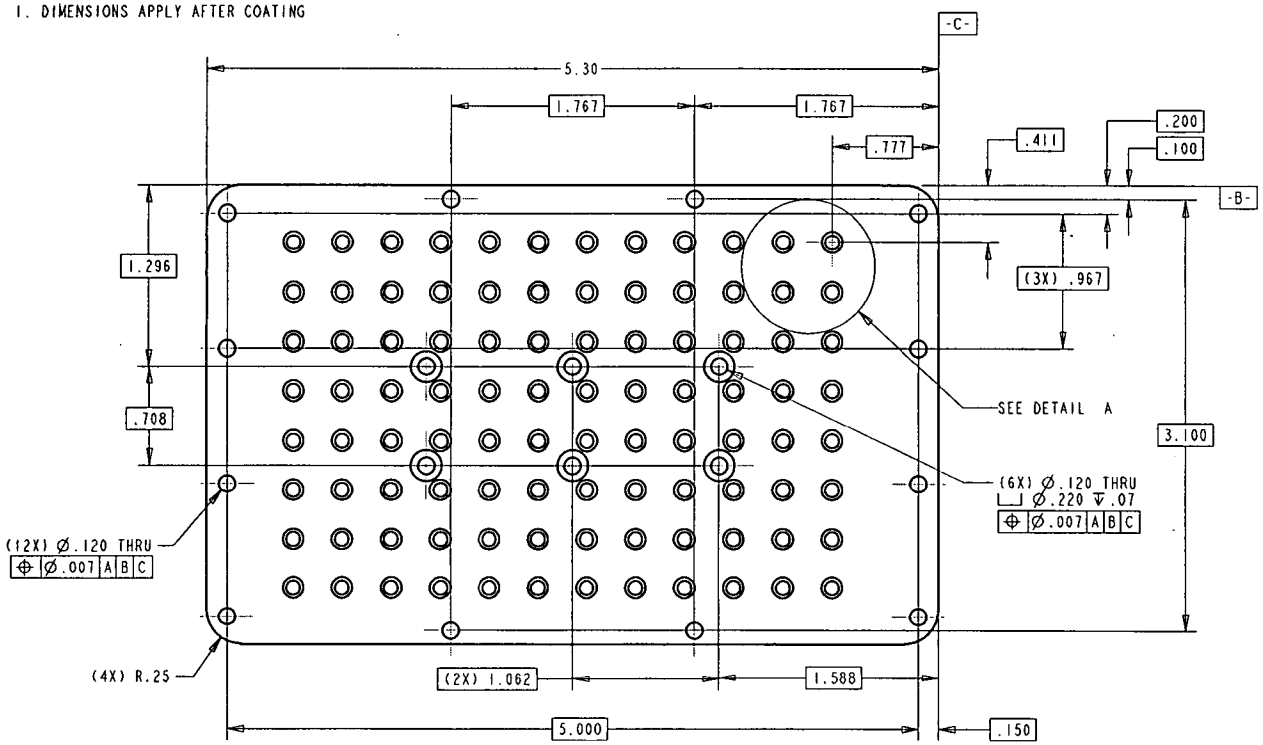
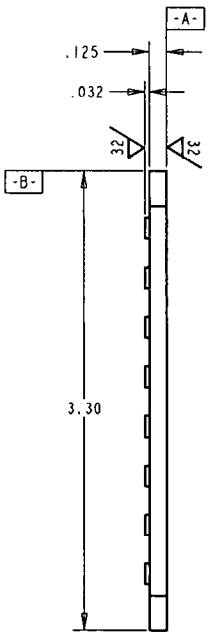
	DESIGN: SCHARFSTEIN — DATE: 4/2003		MICROTITER
MATERIAL: TEFLON	DRAWN: SCHARFSTEIN — DATE: 11/17/03	SIZE: A — ASM. NO.: ISMA-F-0001	REV.: -
FINISH: NONE	APPROVED — DATE	SCALE: 1/2	SHEET: 1 OF 1

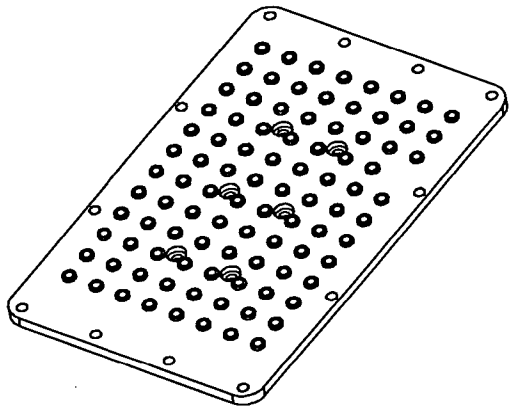
REV.	DESCRIPTION	DATE	APPROVAL

NOTES:

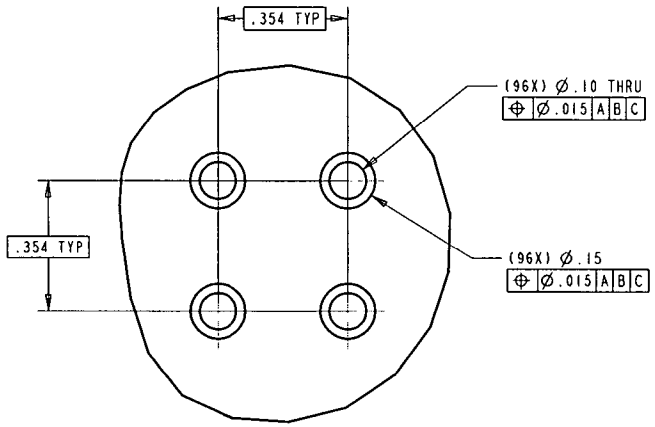
1. DIMENSIONS APPLY AFTER COATING







SCALE 1/2



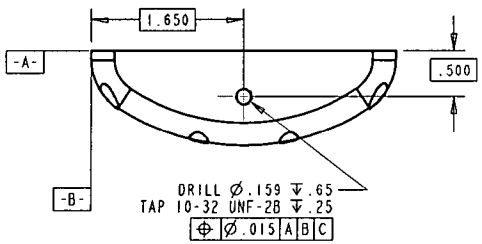
DETAIL A
SCALE 2/1

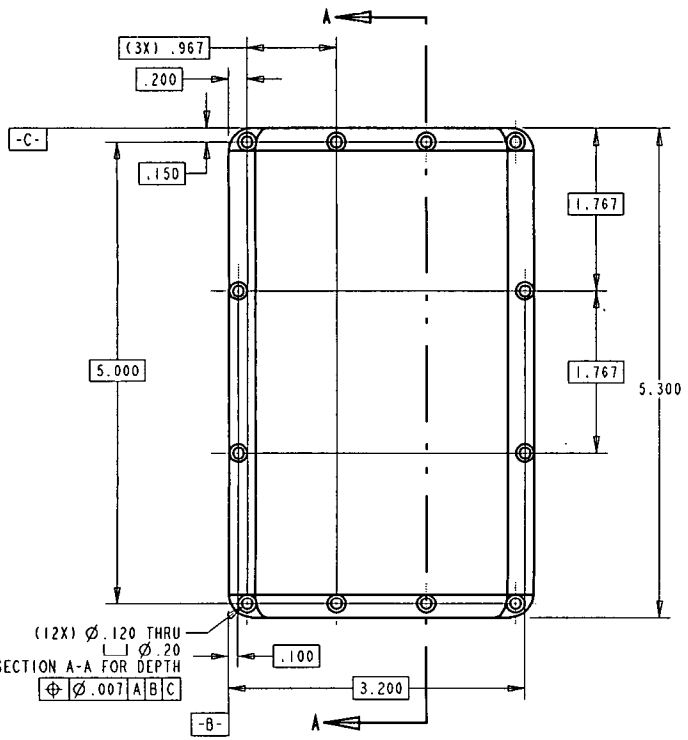
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16, .X ±0.03, .XX ±0.01, .XXX ±0.005, ANGLES ±1/2° SURF. 125	ISMA	IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY, Department of Physics and Astronomy, Baltimore, MD 21218
	DESIGN SCHARFSTEIN / DATE 4/11/03		RESTRICTER PLATE
MATERIAL 300 SERIES SS	DRAWN B. TURSI / DATE 11/13/03	SIZE A / ASM. NO. ISMA-F-0002	REV. -
FINISH VITON	APPROVED / DATE	SCALE 3/4	SHEET 1 OF 1

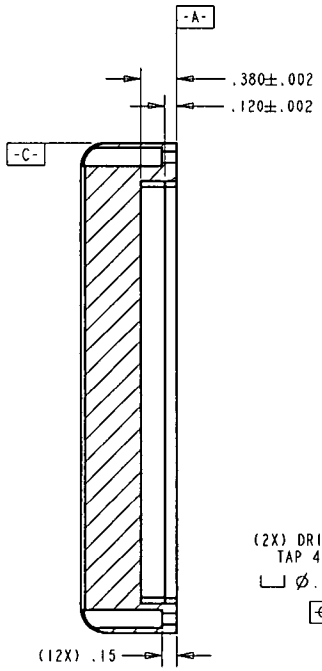
NOTES:

1. DEBUR ALL SHARP EDGES

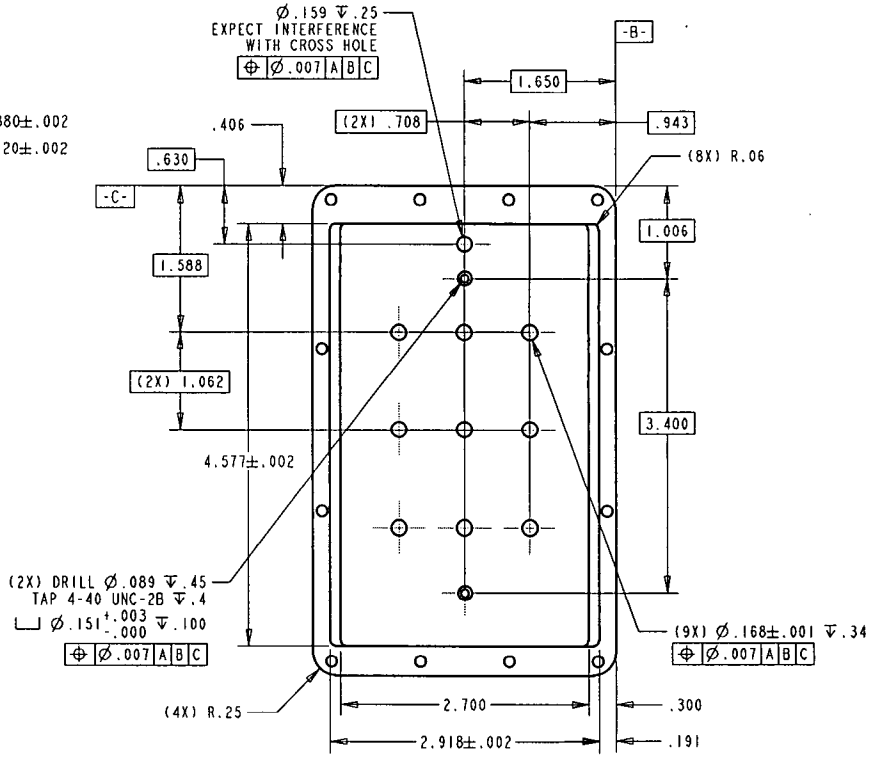
REV.	DESCRIPTION	DATE	APPROVAL

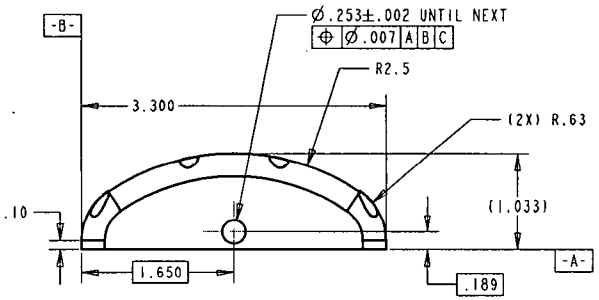






SECTION A-A





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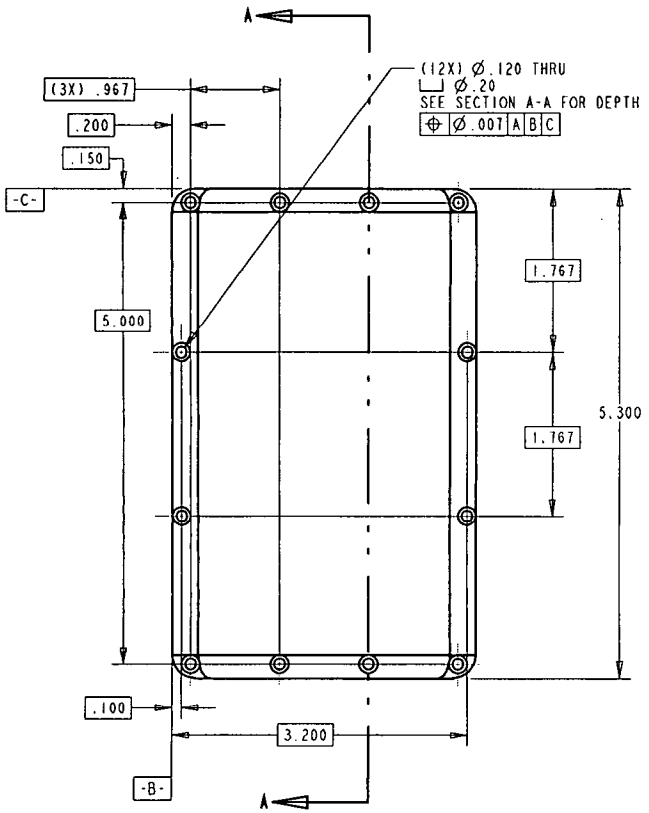
	DESIGN SCHARFSTEIN	DATE 4/2003	INTAKE HOUSING	
MATERIAL DELRIN - BLACK	DRAWN SCHARFSTEIN	DATE 11/18/03	SIZE A	ASM. NO. ISMA-F-0003 REV. -
FINISH NONE	APPROVED	DATE	SCALE 1/2	SHEET 1 OF 1

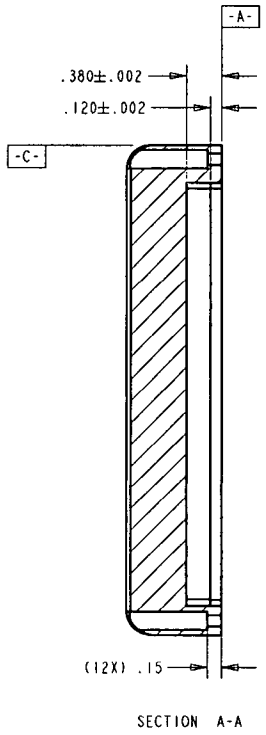
120

REV.	DESCRIPTION	DATE	APPROVAL

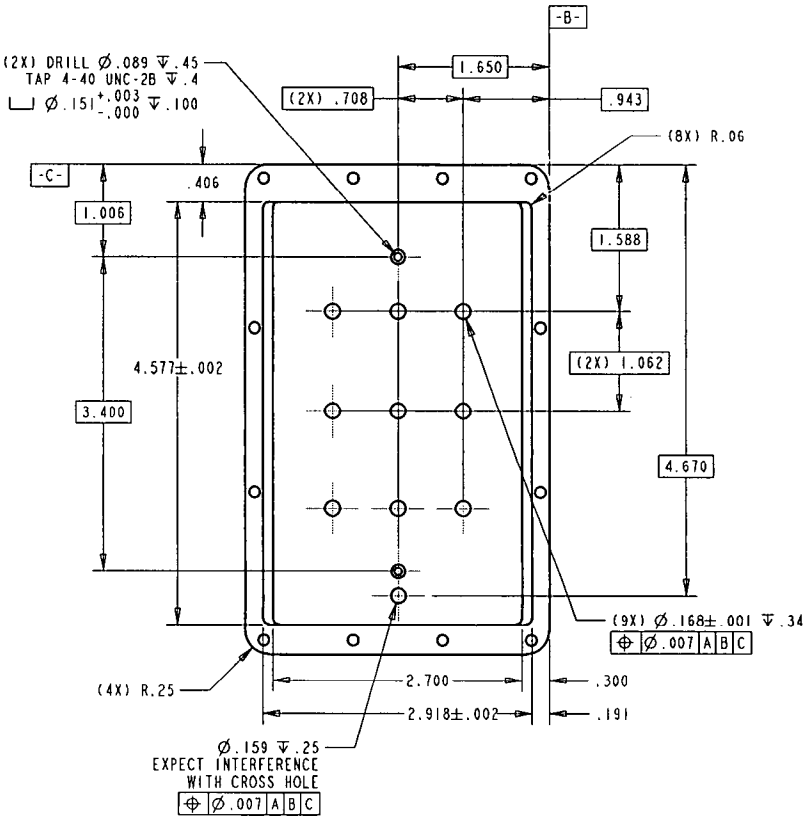
NOTES:

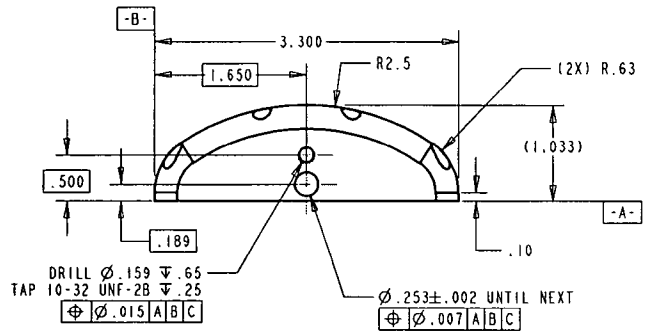
1. DEBUR ALL SHARP EDGES





SECTION A-A





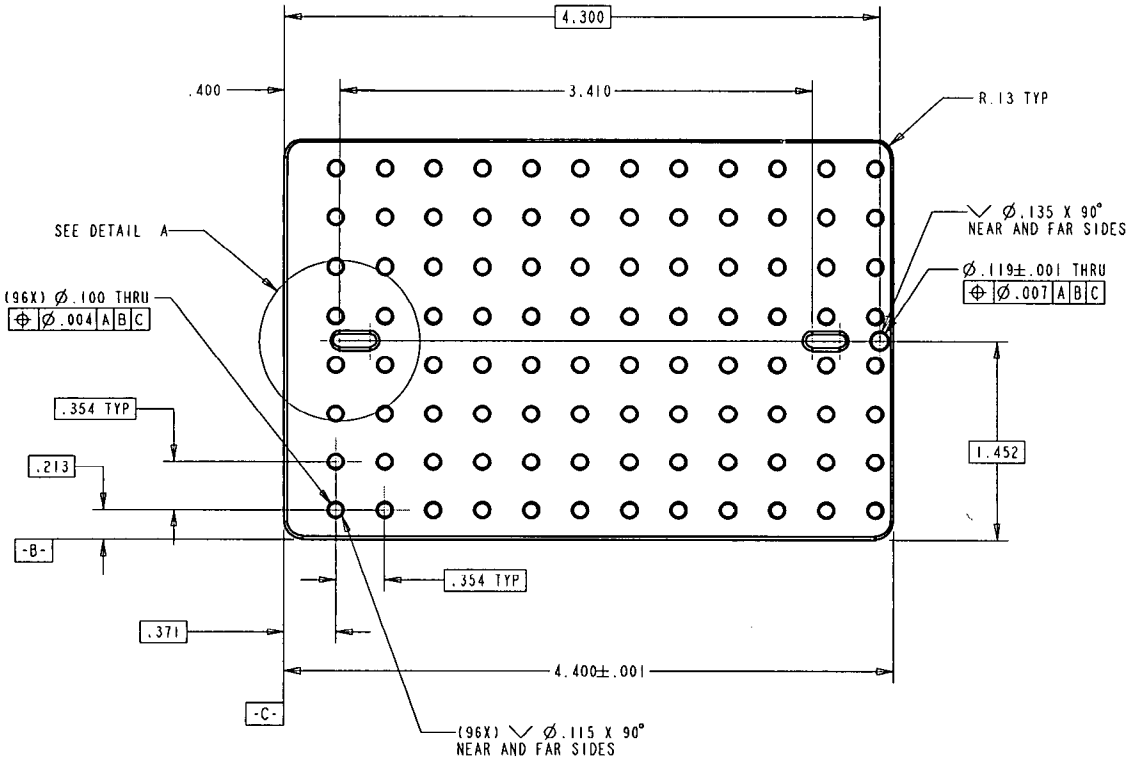
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16 .X ±0.03 .XX ±0.01 .XXX ±0.005 ANGLES ±1/2° SURF. 125	ISMA	IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218
	DESIGN SCHARFSTEIN / DATE 12/3/2003		EXHAUST HOUSING
MATERIAL DELRIN - BLACK	DRAWN SCHARFSTEIN / DATE 12/03/03	SIZE A / ASM. NO. ISMA-F-0004	REV. -
FINISH NONE	APPROVED / DATE	SCALE 1/2	SHEET 1 OF 1

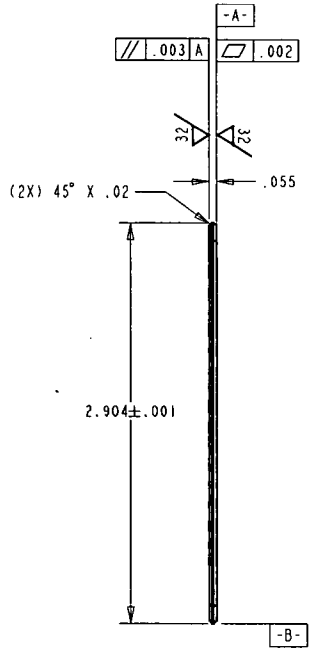
REV.	DESCRIPTION	DATE	APPROVAL

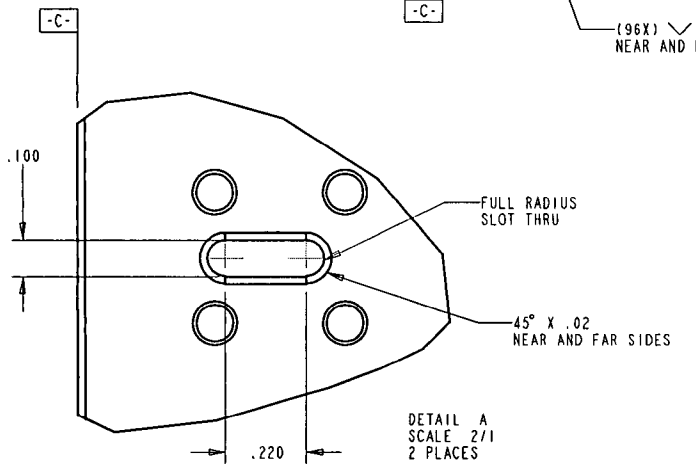
NOTES:

1. DEBUR ALL SHARP EDGES

2 TEFLON COATING ON ALL SURFACES. MACHINE SS TO DIMENSIONS SHOWN ON DRAWING. TEFLON COATING WILL ADD .003-.005 INCH TO EACH SURFACE







DETAIL A
SCALE 2/1
2 PLACES

UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16, .X ±0.03, .XX ±0.01, .XXX ±0.005, ANGLES ±1/2° SURF. 125	ISMA		JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218		
	DESIGN SCHARFSTEIN	DATE 4/11/03	VALVE PLATE		
MATERIAL 300 SERIES SS	DRAWN B. TURSI	DATE 11/11/03	SIZE A	ASM. NO. ISMA-F-0005	REV. -
FINISH 2	APPROVED	DATE	SCALE 3/4	SHEET 1 OF 1	

122

REV.	DESCRIPTION	DATE	APPROVAL

NOTES:

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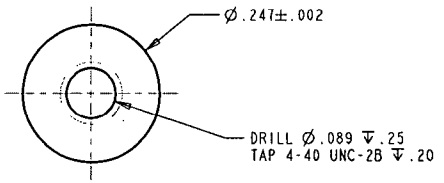

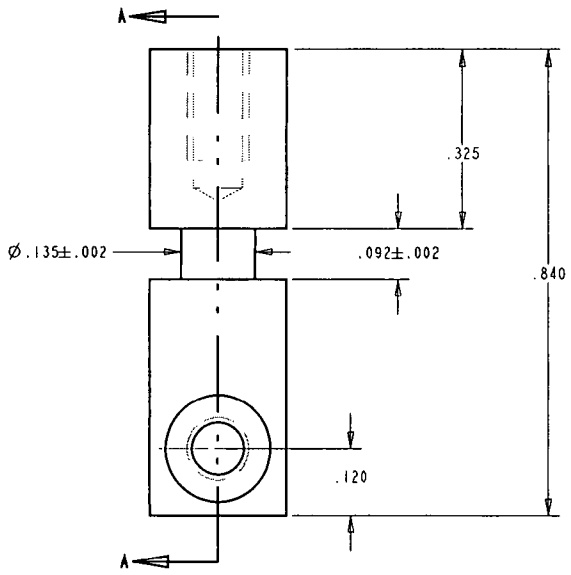

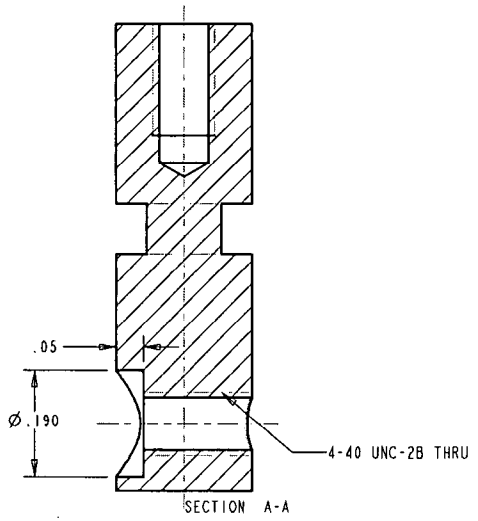

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	DESIGN SCHARFSTEIN	DATE 12/4/2003	VALVE PLATE ARM		
MATERIAL DELRIN - BLACK	DRAWN SCHARFSTEIN	DATE 12/04/03	SIZE A	ASM. NO. ISMA-F-0006	REV. -
FINISH NONE	APPROVED	DATE	SCALE 3/1	SHEET 1 OF 1	

123

REV.	DESCRIPTION	DATE	APPROVAL

NOTES:

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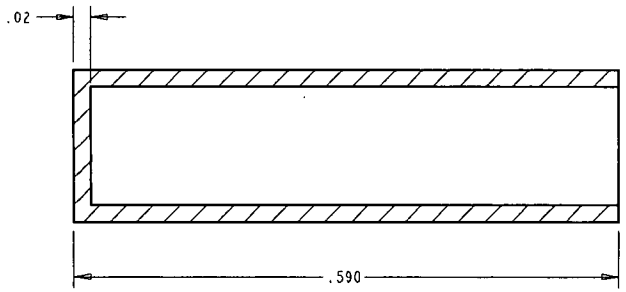
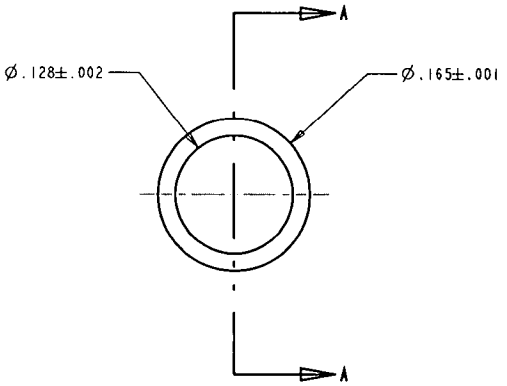


SECTION A-A



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	DESIGN SCHARFSTEIN	DATE 12/4/2003	SPRING SHEATH	
MATERIAL TEFLON	DRAWN SCHARFSTEIN	DATE 12/04/03	SIZE A	ASM. NO. ISMA-F-0007 REV. -
FINISH NONE	APPROVED	DATE	SCALE 5/1	SHEET 1 OF 1

REV.	DESCRIPTION	DATE	APPROVAL

NOTES:

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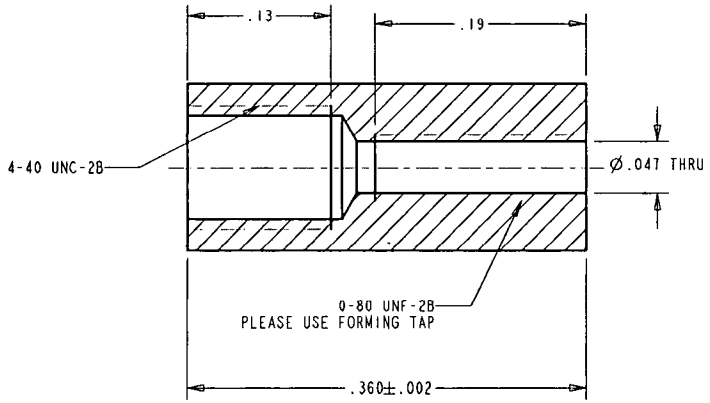
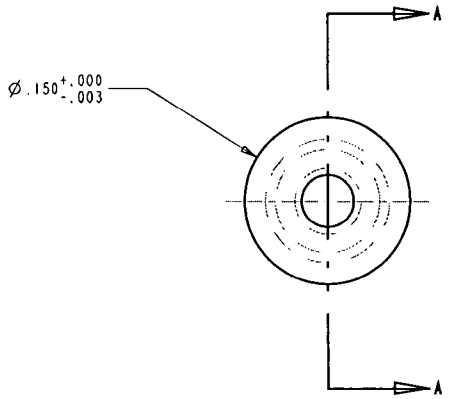


SECTION A-A



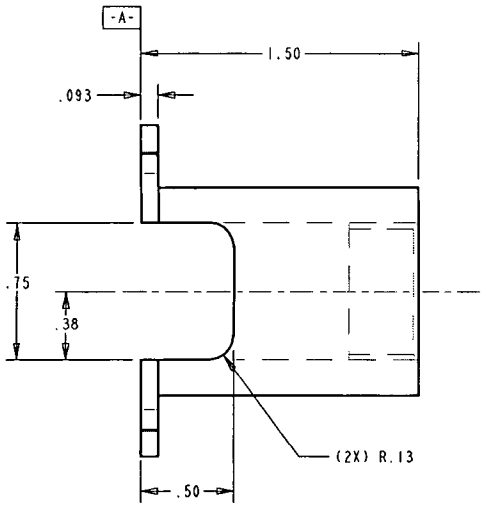
125

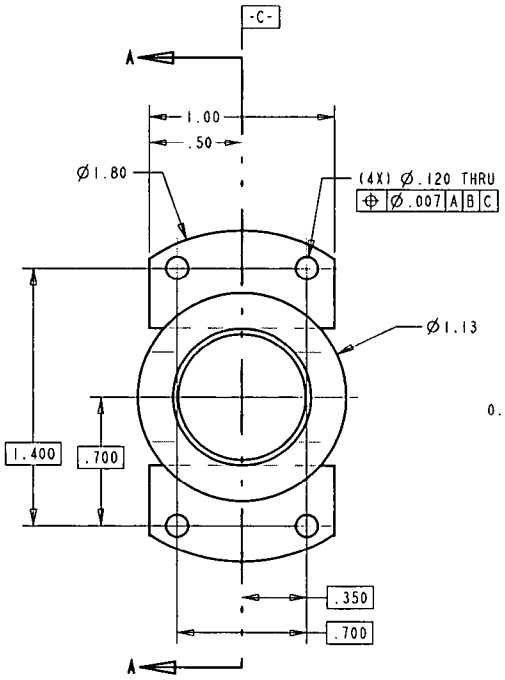
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16, .X ±0.03, .XX ±0.01, .XXX ±0.005, ANGLES ±1/2° SURF. 125	ISMA		IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218
	DESIGN SCHARFSTEIN	DATE 12/4/2003	VALVE PLATE RETAINER	
MATERIAL DELRIN - BLACK	DRAWN SCHARFSTEIN	DATE 12/04/03	SIZE A	ASM. NO. ISMA-F-0008 REV. -
FINISH NONE	APPROVED	DATE	SCALE 6/1	SHEET 1 OF 1

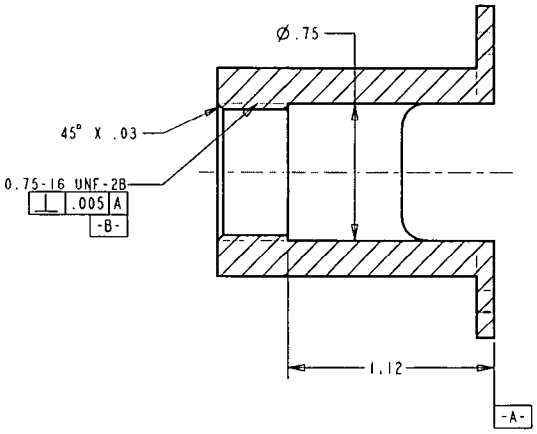
REV.	DESCRIPTION	DATE	APPROVAL

NOTES:

1. DEBUR ALL SHARP EDGES







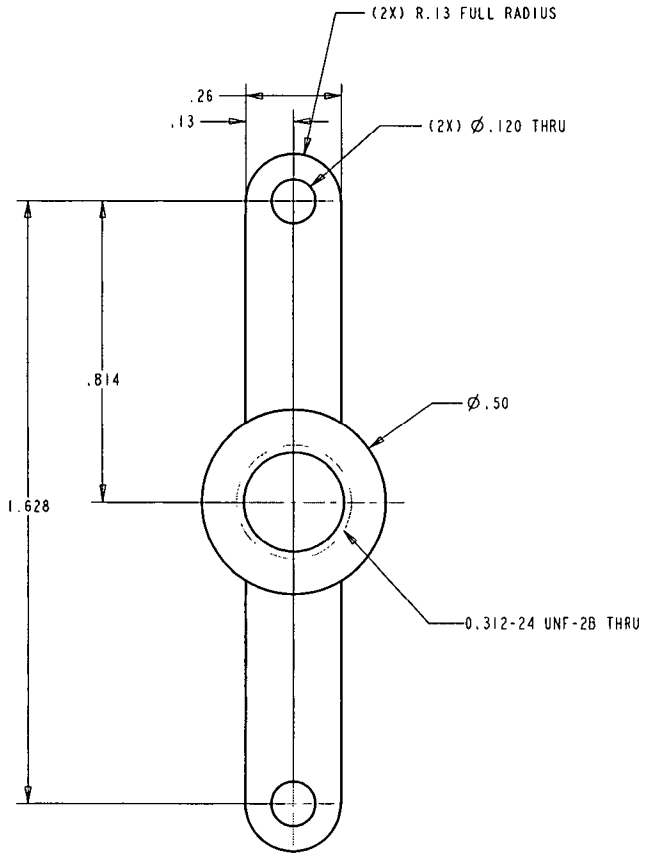
SECTION A-A

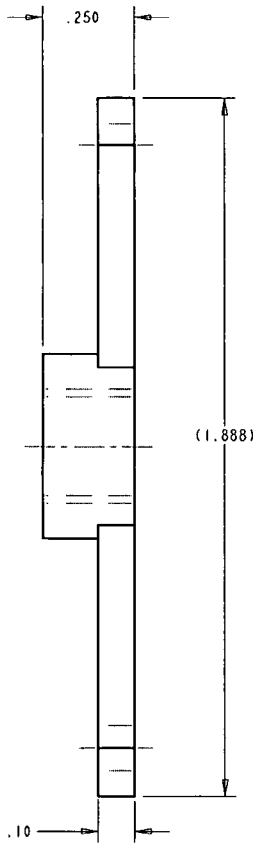
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16 .X ±0.03 .XX ±0.01 .XXX ±0.005 ANGLES ±1/2° SURF. 125	ISMA		JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218
	DESIGN SCHARFSTEIN	DATE 12/4/2003	CLYNDER MOUNT
MATERIAL 300 SERIES SS	DRAWN SCHARFSTEIN	DATE 12/04/03	SIZE A / ASM. NO. ISMA-F-0009 / REV. -
FINISH NONE	APPROVED	DATE	SCALE 1/1 / SHEET 1 OF 1

REV.	DESCRIPTION	DATE	APPROVAL

NOTES:

1. DEBUR ALL SHARP EDGES



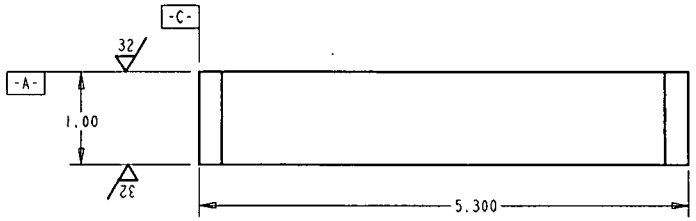


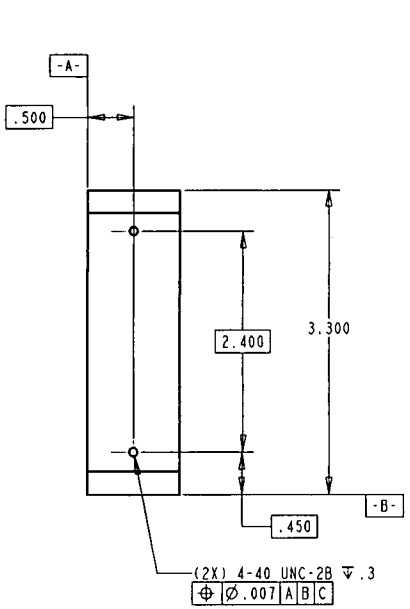
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16 .X ±0.03 .XX ±0.01 .XXX ±0.005 ANGLES ±1/2° SURF. 125	ISMA		JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218		
	DESIGN SCHARFSTEIN	DATE 4/2003	CYLINDER T		
MATERIAL 300 SERIES SS	DRAWN SCHARFSTEIN	DATE 12/16/03	SIZE A	ASM. NO. ISMA-F-0010	REV. -
FINISH NONE	APPROVED	DATE	SCALE 2/1	SHEET 1 OF 1	

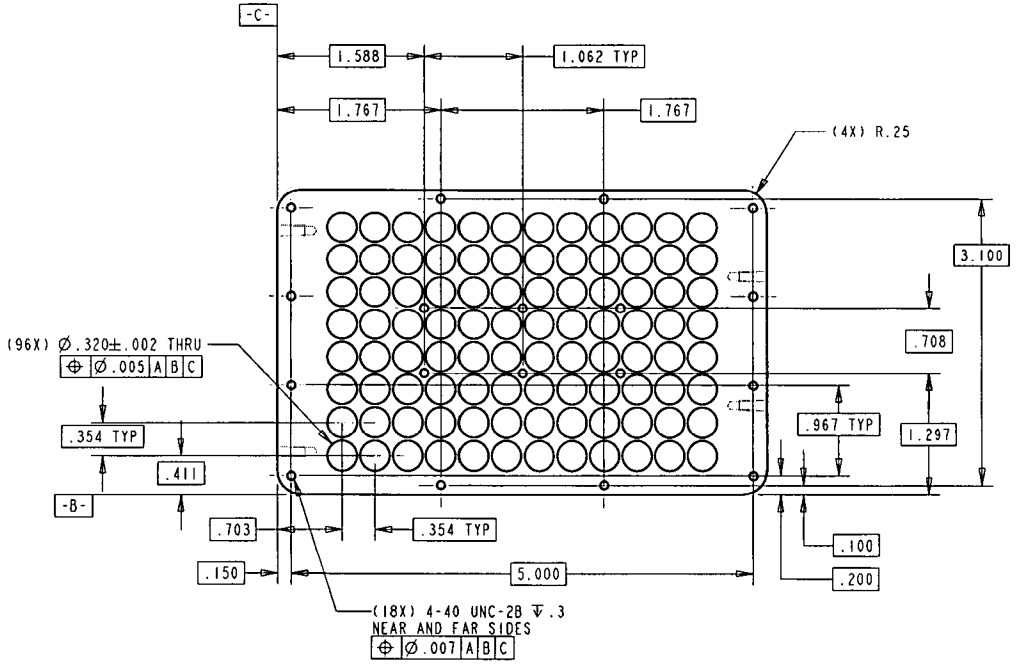
REV.	DESCRIPTION	DATE	APPROVAL
A	INCREASED SIZE OF 96 HOLES & CHANGED TOLERANCE FROM .295 TO .320 ±.002 WITH TRUE POSITION FROM .007 TO .005	1.7.2004	SCHARFSTEIN

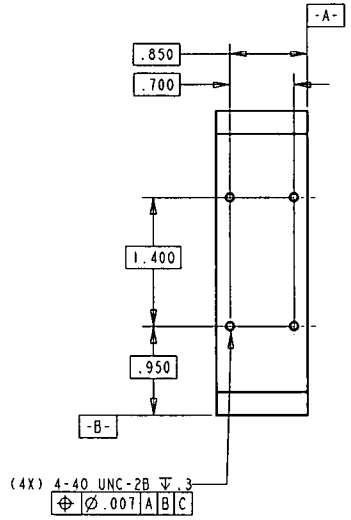
NOTES:

1. DEBUR ALL SHARP EDGES



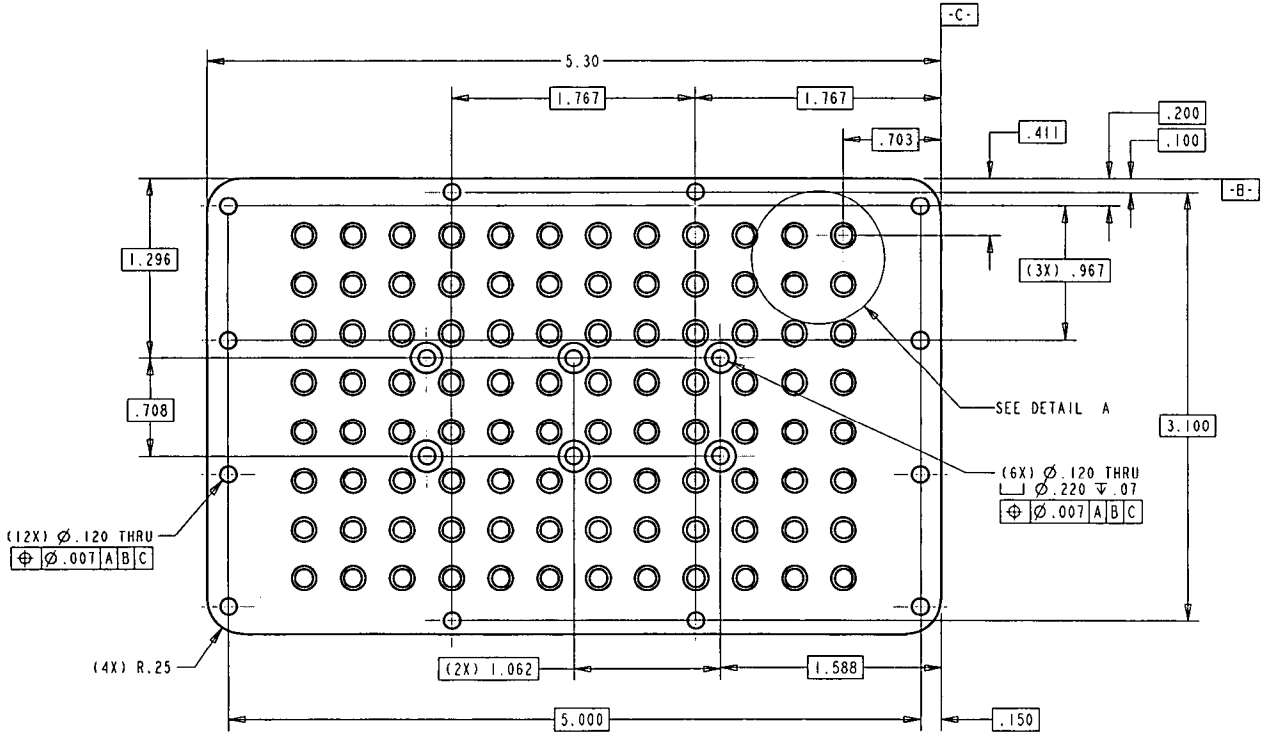


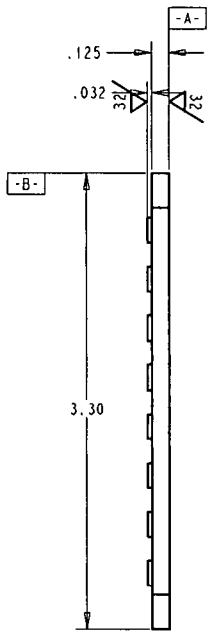


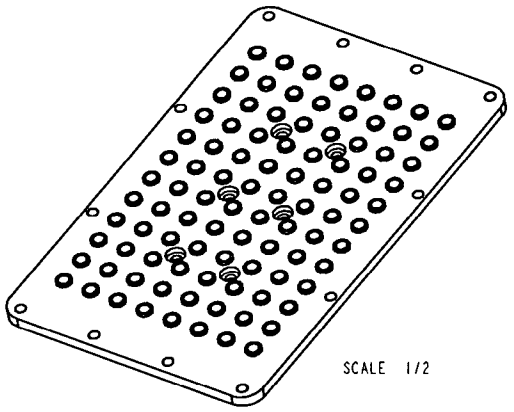


UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16, .X ±0.03, .XX ±0.01, .XXX ±0.005, ANGLES ±1/2° SURF. 125	ISMA		JOHNS HOPKINS UNIVERSITY, Department of Physics and Astronomy, Baltimore, MD 21218		
	DESIGN SCHARFSTEIN	DATE 4/2003	MICROTITER		
MATERIAL TEFLON	DRAWN SCHARFSTEIN	DATE 11/17/03	SIZE A	ASM. NO. ISMA-F-0001	REV. A
FINISH NONE	APPROVED	DATE	SCALE 1/2	SHEET 1 OF 1	

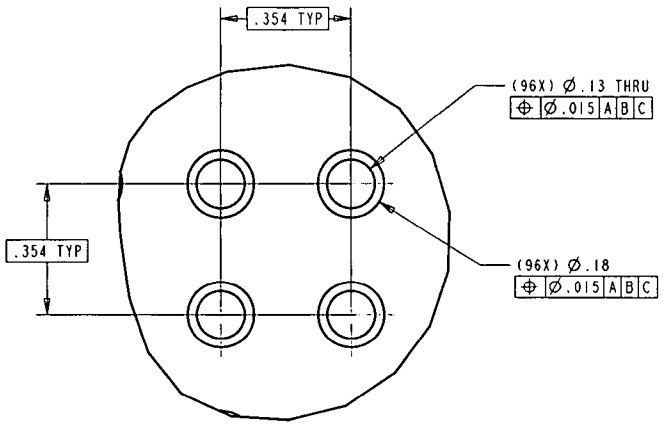
REV.	DESCRIPTION	DATE	APPROVAL
A	MOVED LOCATION OF 96 HOLES .074 TO THE CENTER OF THE HOLES OF ISMA-F-0001	1.5.2004	SCHARFSTEIN
B	INCREASED SIZE OF 96 HOLES, PROTRUSIONS FROM .10, .15 TO .13, .18 RESPECTIVELY	1.6.2004	SCHARFSTEIN







SCALE 1/2



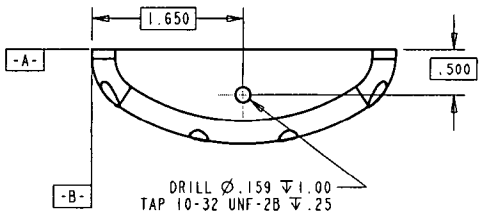
DETAIL A
SCALE 2/1

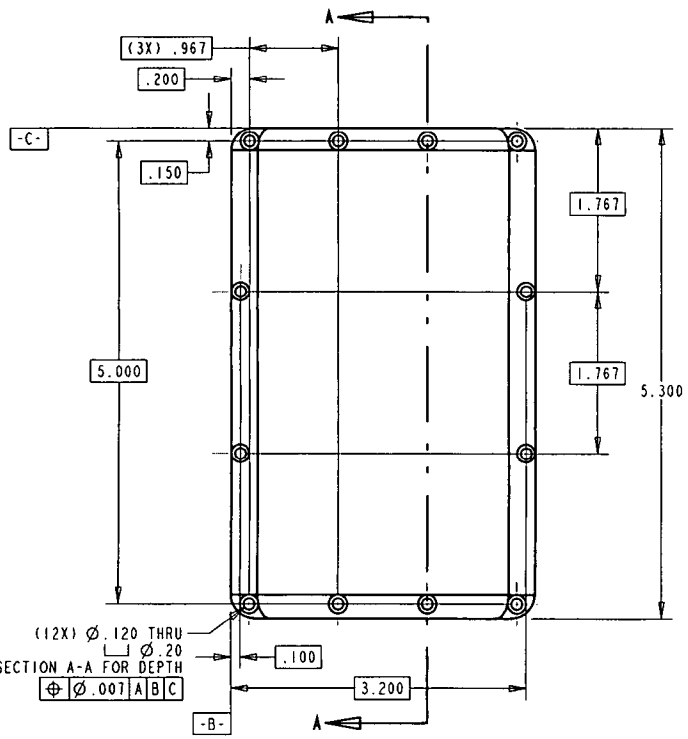
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16, .X ±0.03, .XX ±0.01, .XXX ±0.005, ANGLES ±1/2° SURF. 125	ISMA	IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218	
	DESIGN SCHARFSTEIN	DATE 4/11/03	RESTRICTER PLATE	
MATERIAL VITON	DRAWN B. TURSI	DATE 11/13/03	SIZE A	ASM. NO. ISMA-F-0002 REV. B
FINISH NONE	APPROVED	DATE	SCALE 3/4	SHEET 1 OF 1

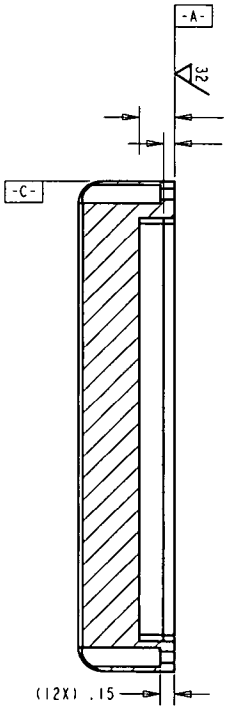
NOTES:

1. DEBUR ALL SHARP EDGES

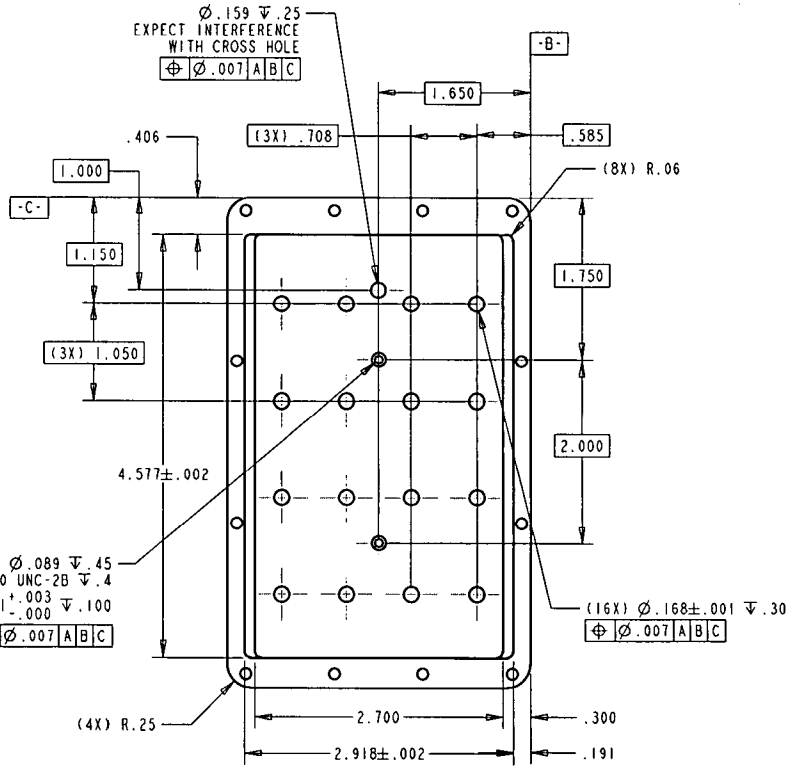
REV.	DESCRIPTION	DATE	APPROVAL
A	ADDED 7 BLIND .168 DIA HOLES AND DECREASED DEPTH FROM .34 TO .30 ADDED 32-SURF FIN TO -A- MOVED C-BORED, 4-40 TAPPED HOLES INCREASED DEPTH OF .159 DIA HOLE FROM .65 TO 1.00 CHANGED COLOR OF DELRIN FROM BLACK TO WHITE	1.7.2004	SCHARFSTEIN

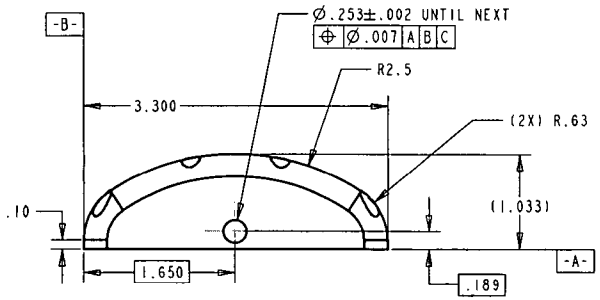






SECTION A-A



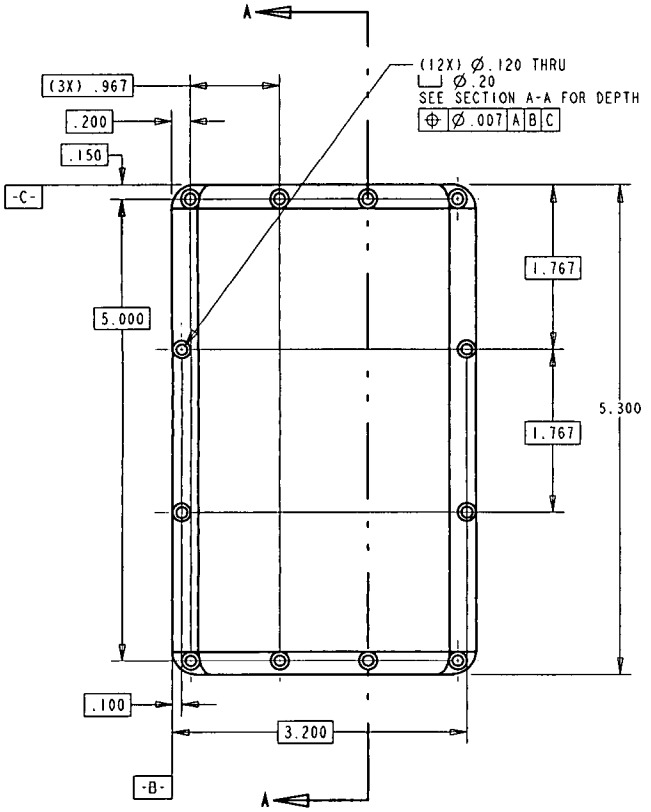


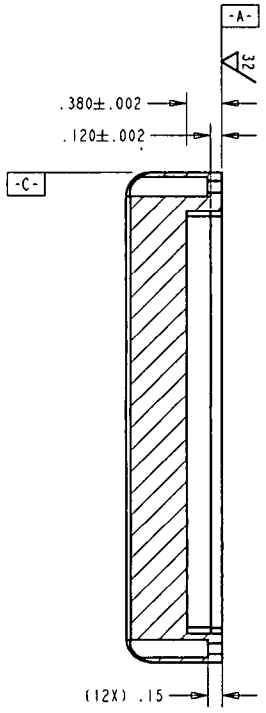
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16 .X ±0.03 .XX ±0.01 .XXX ±0.005 ANGLES ±1/2° SURF. 125	ISMA	IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218
	DESIGN SCHARFSTEIN / DATE 4/2003		INTAKE HOUSING
MATERIAL DELRIN - WHITE	DRAWN SCHARFSTEIN / DATE 11/18/03	SIZE A	ASM. NO. ISMA-F-0003 / REV. A
FINISH NONE	APPROVED / DATE	SCALE 1/2	SHEET 1 OF 1

REV.	DESCRIPTION	DATE	APPROVAL
A	ADDED 7 BLIND .168 DIA HOLES AND DECREASED DEPTH FROM .34 TO .30 ADDED 32-SURF FIN TO -A- MOVED C-BORED, 4-40 TAPPED HOLES INCREASED DEPTH OF .159 DIA HOLE FROM .65 TO 1.00 CHANGED COLOR OR DELRIN FROM BLACK TO WHITE	1.7.2004	SCHARFSTEIN

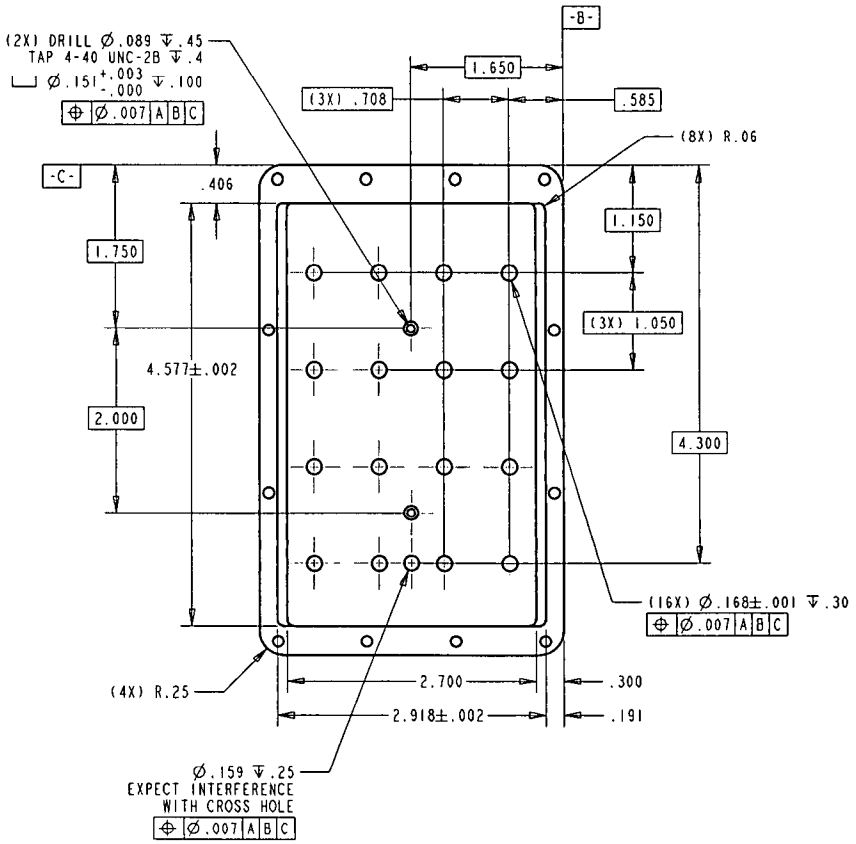
NOTES:

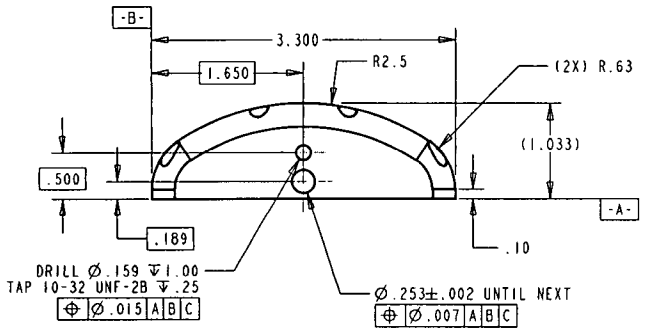
1. DEBUR ALL SHARP EDGES





SECTION A-A





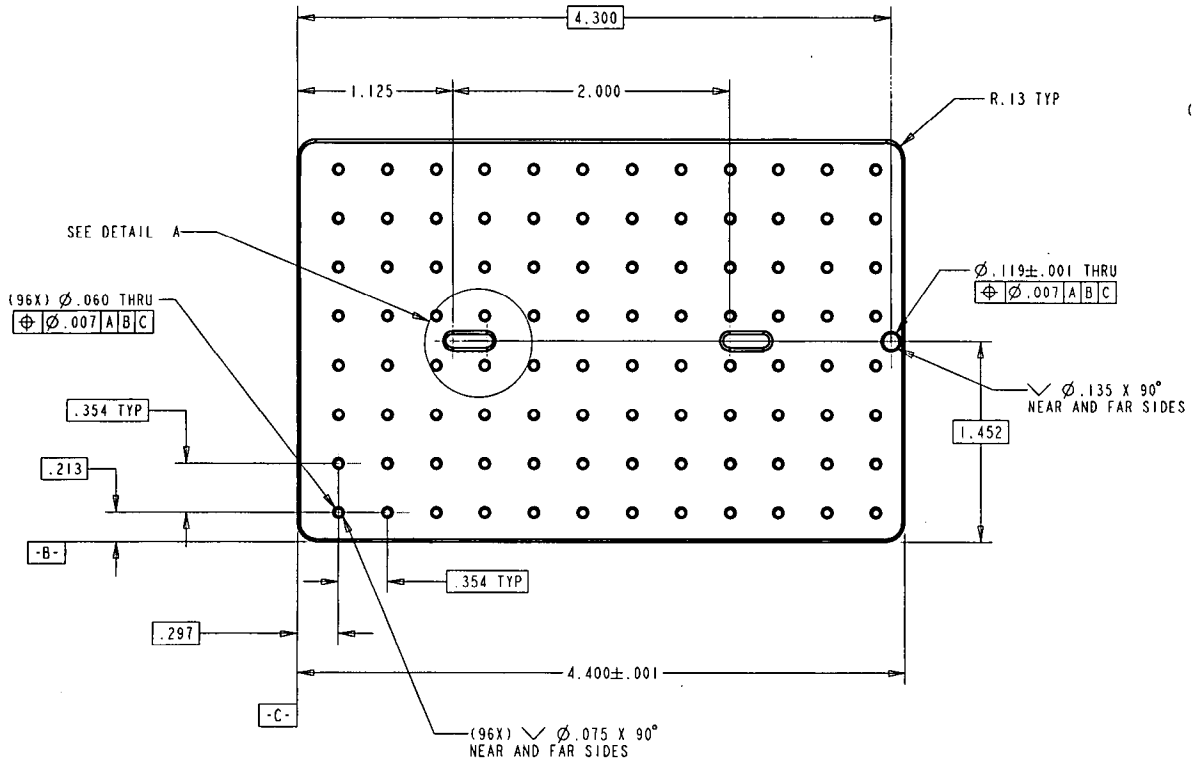
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16 .X ±0.03 .XX ±0.01 .XXX ±0.005 ANGLES ±1/2° SURF. 125	ISMA	IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218
	DESIGN SCHARFSTEIN / DATE 12/3/2003	EXHAUST HOUSING	
MATERIAL DELRIN - WHITE	DRAWN SCHARFSTEIN / DATE 12/03/03	SIZE A / ASM. NO. ISMA-F-0004	REV. A
FINISH NONE	APPROVED / DATE	SCALE 1/2	SHEET 1 OF 1

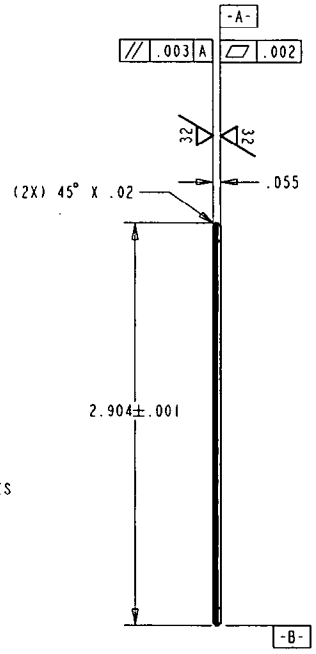
NOTES:

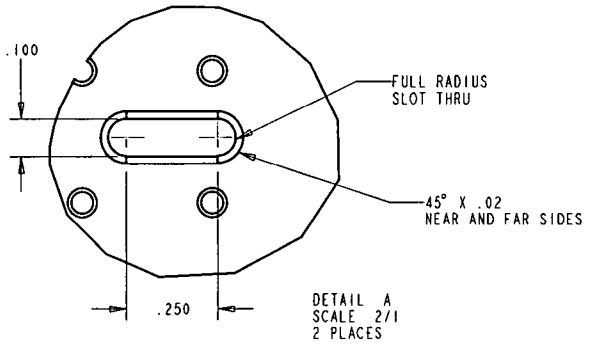
1. DEBUR ALL SHARP EDGES

2 TEFLON COATING ON ALL SURFACES. MACHINE SS TO DIMENSIONS SHOWN ON DRAWING. TEFLON COATING WILL ADD .003-.005 INCH TO EACH SURFACE

REV.	DESCRIPTION	DATE	APPROVAL
A	MOVED 96 HOLES .074 TO CENTER OF HOLES IN ISMA-F-0001 DECREASED SIZE OF 96 HOLES AND CHANGED GEOM. TOL. FROM .100 TO .060 AND TRUE POSITION FROM .004 TO .007 MOVED SLOTS CLOSER TO MIDDLE AND INCREASED SLOT LENGTH FROM .220 TO .250	1.6.2004	SCHARFSTEIN







UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16 .X ±0.03 .XX ±0.01 .XXX ±0.005 ANGLES ±1/2° SURF. 125	ISMA	IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218
	DESIGN SCHARFSTEIN / DATE 4/11/03		VALVE PLATE
MATERIAL 300 SERIES SS	DRAWN B. TURSI / DATE 11/11/03	SIZE A / ASM. NO. ISMA-F-0005	REV. A
FINISH 2	APPROVED / DATE	SCALE 3/4	SHEET 1 OF 1

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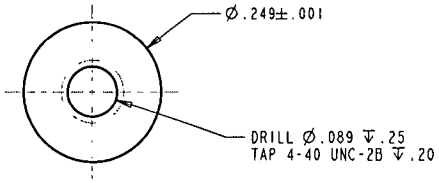
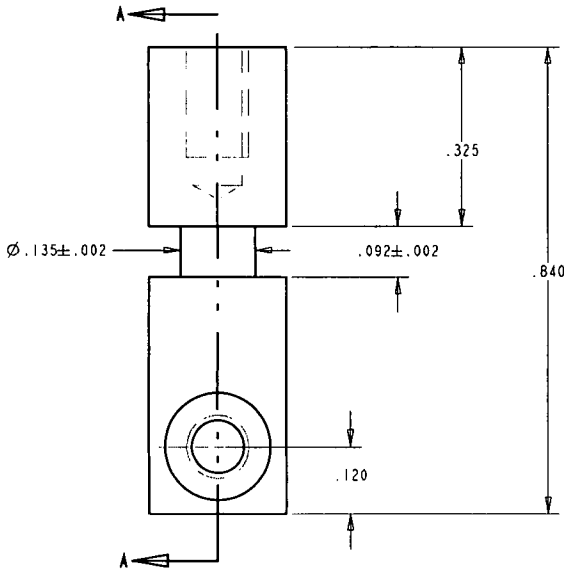
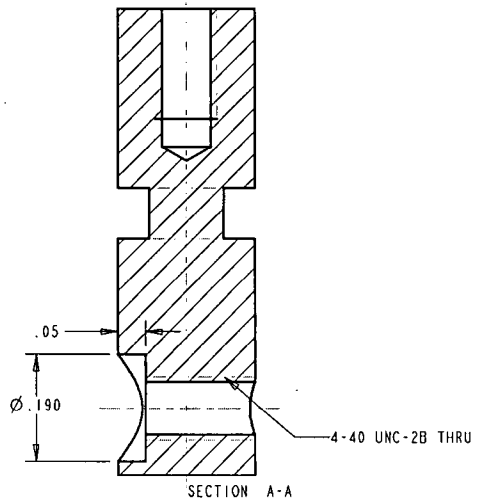
REV.	DESCRIPTION	DATE	APPROVAL
A	INCREASED OUTSIDE DIA FROM .247 +/-.002 TO .249 +/-.001 CHANGED COLOR OF DELRIN FROM BLACK TO WHITE	1.7.2004	SCHARFSTEIN

NOTES:

1. DEBUR ALL SHARP EDGES







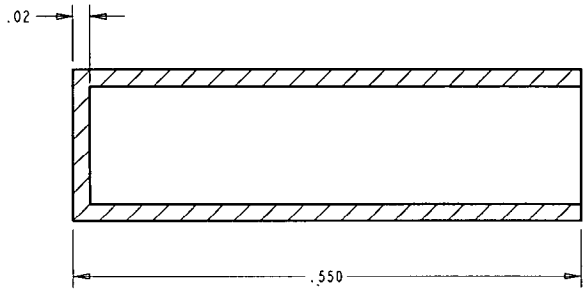
SECTION A-A

UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16, .X ±0.03, .XX ±0.01, .XXX ±0.005, ANGLES ±1/2° SURF. 125	ISMA		JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218		
	DESIGN SCHARFSTEIN	DATE 12/4/2003	VALVE PLATE ARM		
MATERIAL DELRIN - WHITE	DRAWN SCHARFSTEIN	DATE 12/04/03	SIZE A	ASM. NO. ISMA-F-0006	REV. A
FINISH NONE	APPROVED	DATE	SCALE 3/1	SHEET 1 OF 1	

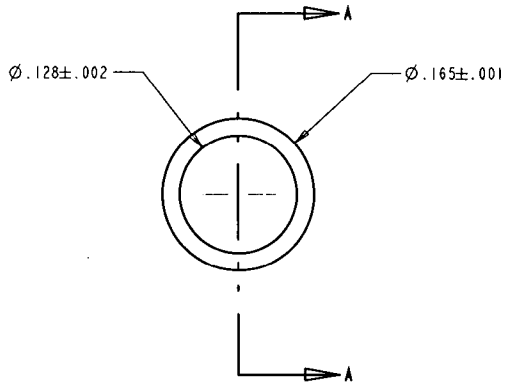
REV.	DESCRIPTION	DATE	APPROVAL
A	CHANGED LENGTH FROM .590 TO .550	1.5.2004	SCHARFSTEIN

NOTES:

1. DEBUR ALL SHARP EDGES



SECTION A-A

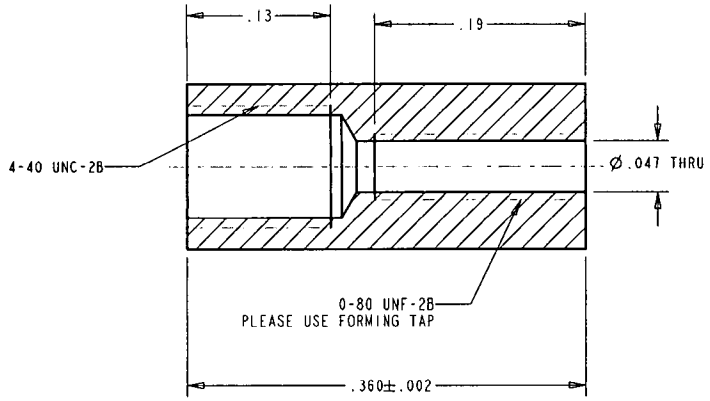


UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16 .X ±0.03 .XX ±0.01 .XXX ±0.005 ANGLES ±1/2° SURF. 125	ISMA		JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218
	DESIGN SCHARFSTEIN	DATE 12/4/2003	SPRING SHEATH
MATERIAL TEFLON	DRAWN SCHARFSTEIN	DATE 12/04/03	SIZE A / ASM. NO. ISMA-F-0007 / REV. A
FINISH NONE	APPROVED	DATE	SCALE 5/1 / SHEET 1 OF 1

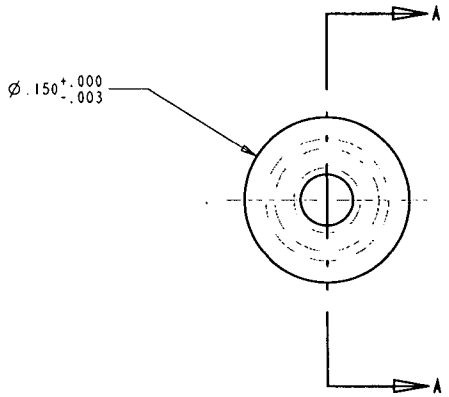
REV.	DESCRIPTION	DATE	APPROVAL
A	CHANGED COLOR OF DELRIN FROM BLACK TO WHITE	1.7.2004	SCHARFSTEIN

NOTES:

1. DEBUR ALL SHARP EDGES



SECTION A-A



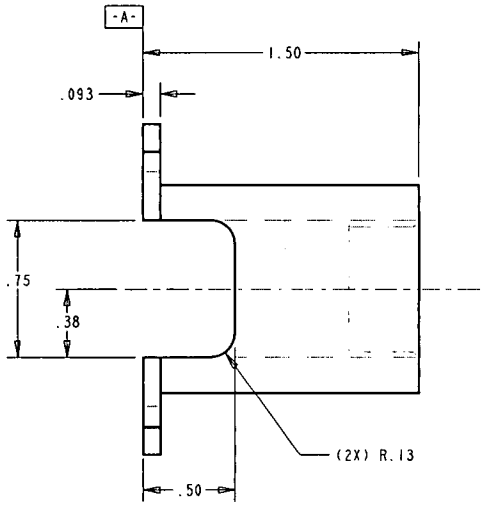
135

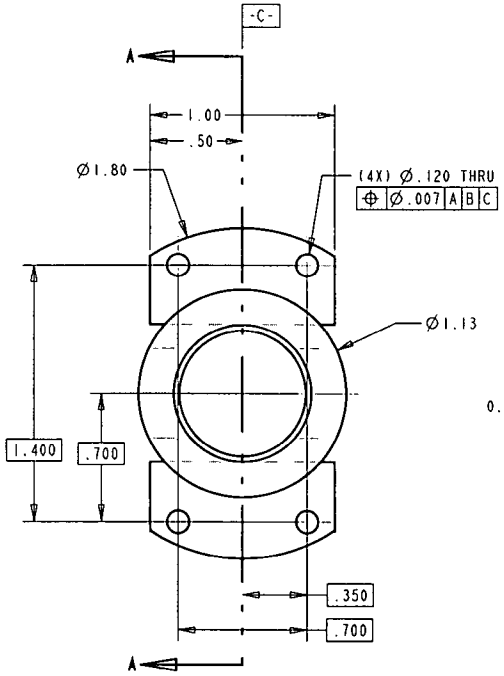
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16, .X ±0.03, .XX ±0.01, .XXX ±0.005, ANGLES ±1/2° SURF. 125	ISMA	IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY, Department of Physics and Astronomy, Baltimore, MD 21218
	DESIGN SCHARFSTEIN / DATE 12/4/2003	VALVE PLATE RETAINER	
MATERIAL DELRIN - WHITE	DRAWN SCHARFSTEIN / DATE 12/04/03	SIZE A / ASM. NO. ISMA-F-0008 / REV. -	
FINISH NONE	APPROVED / DATE	SCALE 6/1 / SHEET 1 OF 1	

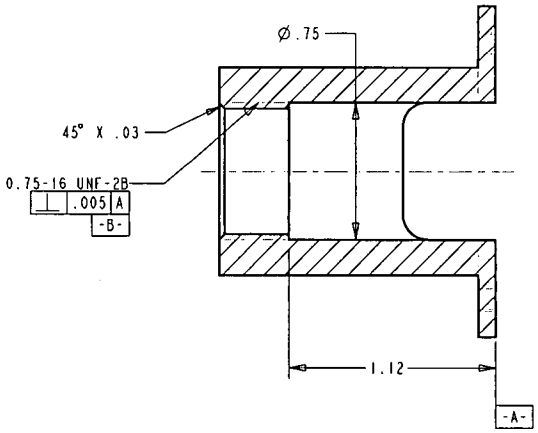
REV.	DESCRIPTION	DATE	APPROVAL

NOTES:

1. DEBUR ALL SHARP EDGES







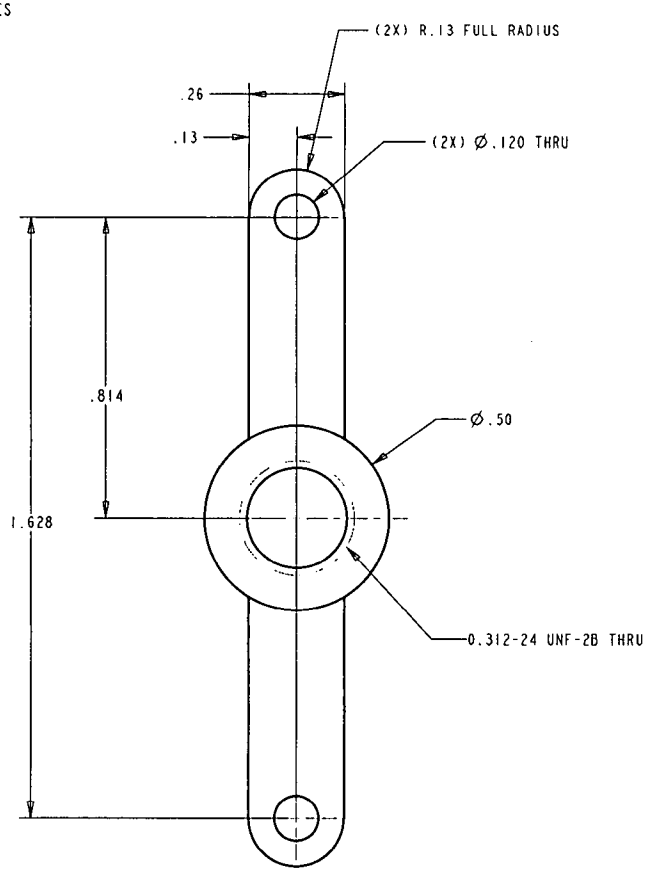
SECTION A-A

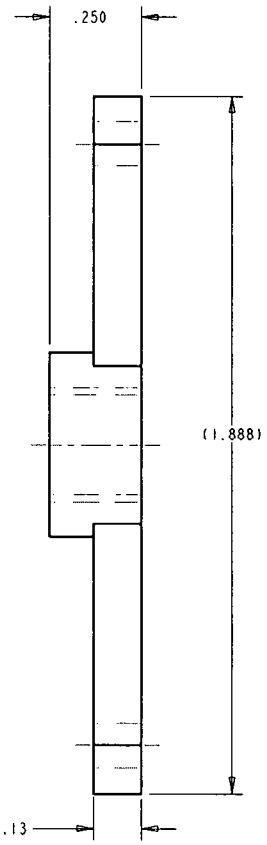
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16, .X ±0.03, .XX ±0.01, .XXX ±0.005, ANGLES ±1/2° SURF. 125	ISMA	IDG Instrument Development Group — JOHNS HOPKINS UNIVERSITY, Department of Physics and Astronomy, Baltimore, MD 21218	
	DESIGN SCHARFSTEIN	DATE 12/4/2003	CLYNDER MOUNT
MATERIAL 300 SERIES SS	DRAWN SCHARFSTEIN	DATE 12/04/03	SIZE A; ASM. NO. ISMA-F-0009; REV. -
FINISH NONE	APPROVED	DATE	SCALE 1/1; SHEET 1 OF 1

REV.	DESCRIPTION	DATE	APPROVAL
A	INCREASED THICKNESS FROM .10 TO .13	1.7.2004	SCHARFSTEIN

NOTES:

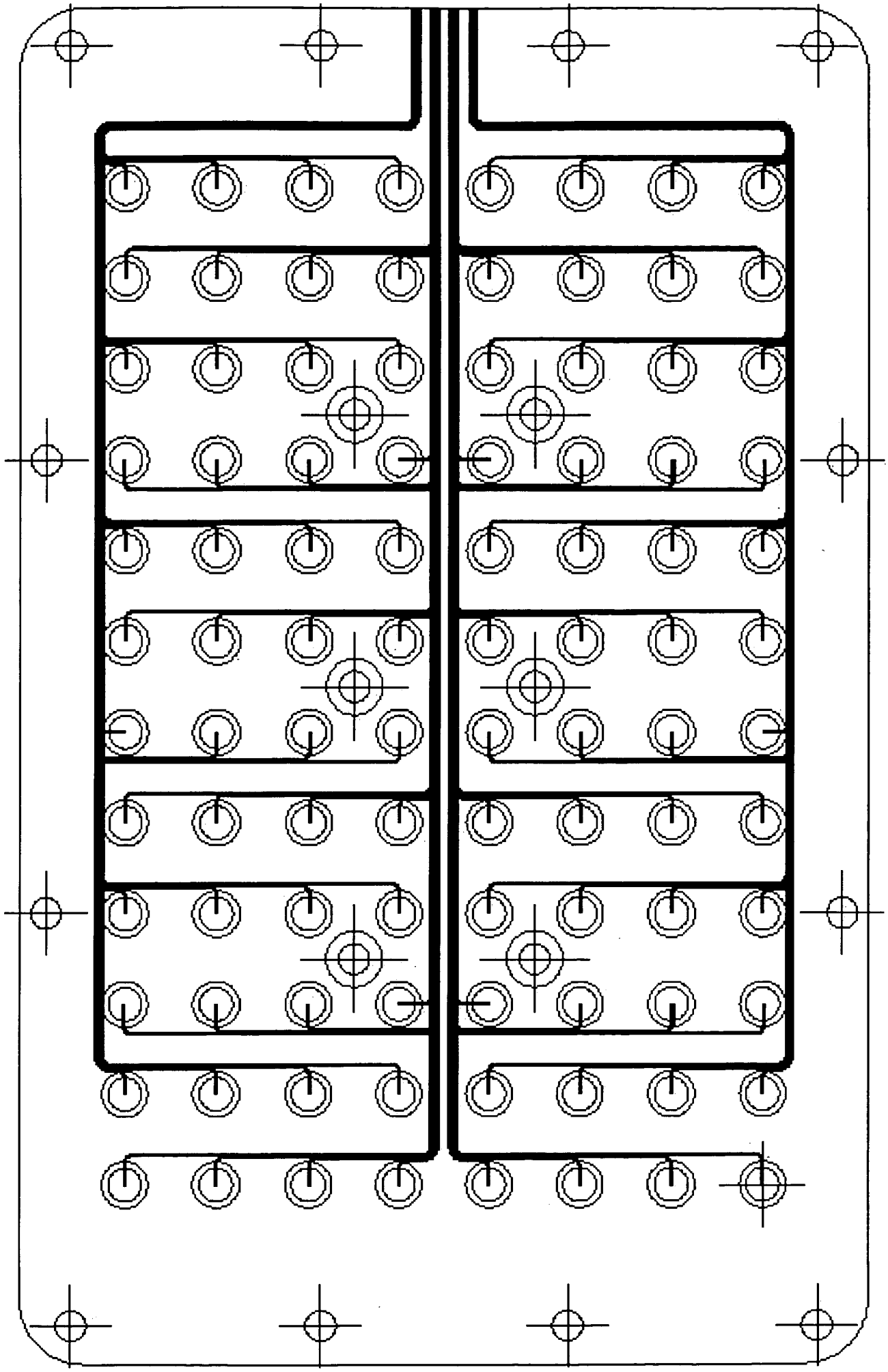
1. DEBUR ALL SHARP EDGES

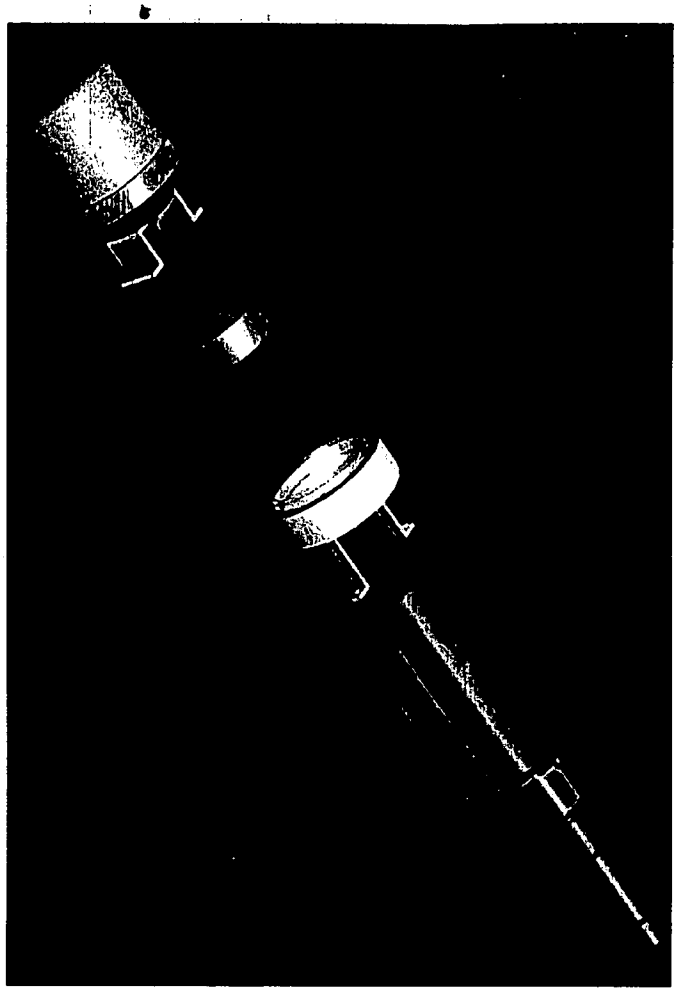


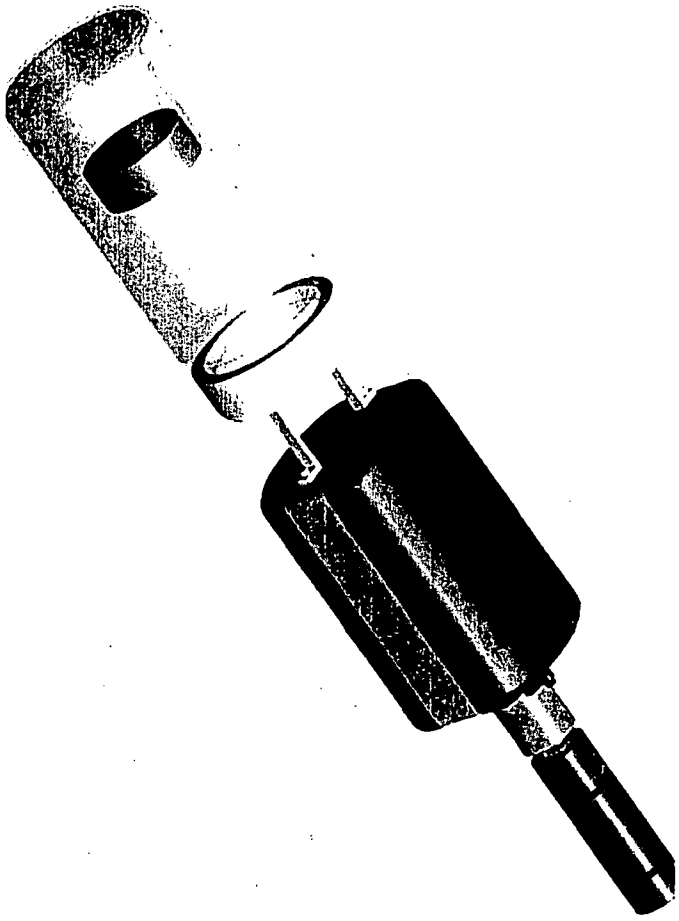


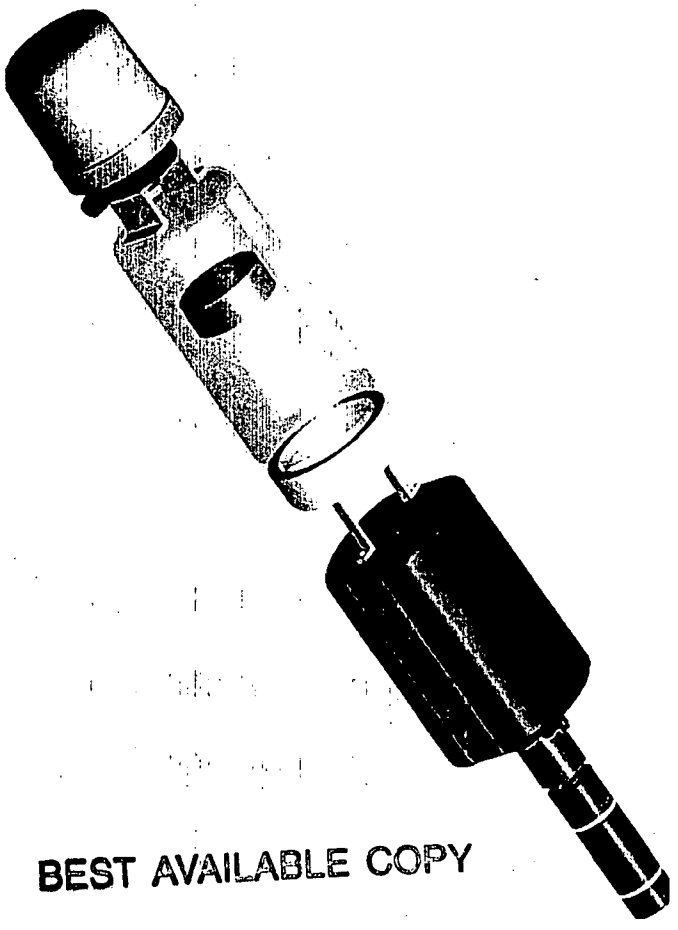
137

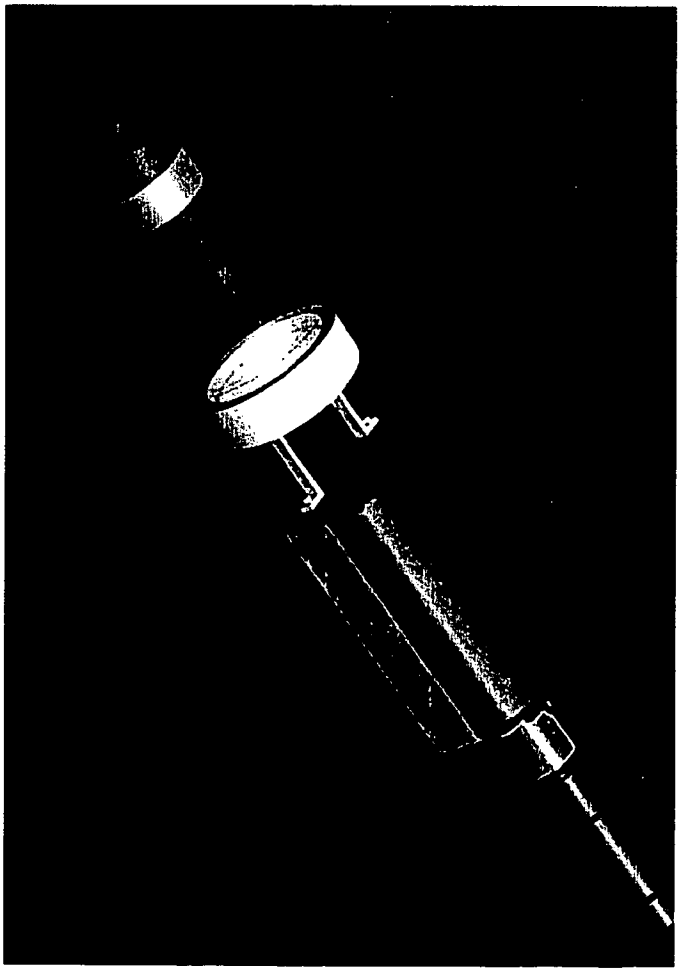
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES TOLERANCES X/XX ±1/16 .X ±0.03 .XX ±0.01 .XXX ±0.005 ANGLES ±1/2° SURF. 125	ISMA	IDG INSTRUMENT DEVELOPMENT GROUP	JOHNS HOPKINS UNIVERSITY Department of Physics and Astronomy Baltimore, MD 21218
	DESIGN SCHARFSTEIN / DATE 4/2003	CYLINDER T	
MATERIAL 300 SERIES SS	DRAWN SCHARFSTEIN / DATE 12/16/03	SIZE A / ASM. NO. ISMA-F-0010	REV. A
FINISH NONE	APPROVED / DATE	SCALE 2/1	SHEET 1 OF 1











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